## Remarks

Reconsideration and withdrawal of the rejections of the claims, in view of the amendments and remarks herein, is respectfully requested. Claims 77-82 and 84-85 are amended, and claims 88-90 are added; as a result, claims 77-90 are now pending in this application. The amendments are intended to advance the application and are not intended to concede to the correctness of the Examiner's position or to prejudice the prosecution of the claims prior to amendment, which claims are present in a continuation of the present application.

The specification is amended at pages 1, 3, 13-14, and 43 to address the Examiner's comments at pages 2-3 of the Office Action.

*The Obviousness-Type Double Patenting Rejection*

The Examiner rejected claims 77-85 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-10 of U.S. Patent No. 6,287,863. This rejection, as it may be maintained with respect to the pending claims, is respectfully traversed.

Claims 1-10 of the '863 patent do not disclose or suggest introducing a cell transformed with a retrotransposon-based vector into an animal, e.g., an organ, tissue or embryo of an animal (claims 77-80, 82-83 and 88), introducing a retrotransposon-based vector into an embryonic stem cell (claim 84), using a retrotransposon-based vector to introduce a gene having an open reading frame flanked at the 3' end by a polyd(T) tract to a cell (claim 81), or using a retrotransposon-based vector to introduce an autonomously replicating DNA sequence to a cell (claim 85).

Accordingly, withdrawal of the obviousness-type double patenting rejection is respectfully requested.

*The 35 U.S.C. § 112, First Paragraph, Rejections*

The Examiner rejected claims 77-85 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. As this rejection may be maintained with respect to the pending claims, it is respectfully traversed.

Specifically, the Examiner asserts that the following phrases are not supported by the specification: i) "expressing a DNA sequence into an animal" in claims 77 and 80; ii) "from step

(d)(1)" in claims 77, 80-82, 84, and 85; iii) "introducing the transformed donor cell to an organ of an animal, tissue of an animal, an embryo of an animal or an animal" in claims 77, 80 and 82; iv) "identifying a transformed cell which contains the DNA sequence of (a)(vi)" in claims 77, 80 and 85; v) "wherein the donor cell is ... or an embryo" in claim 78; vi) "capable of packaging nucleic acid molecules into a virion to yield a transformed donor cell" in claim 80; vii) "producing a double-stranded cDNA containing a gene which is capable of homologous recombination with the genome of a cell" in claim 81; viii) "inserted into the vector 3' of the transcription initiation site...with the genome of the cell" in claim 81; ix) "identifying a cell which contains an integrated form of the vector...which is capable of homologous recombination with the genome of the cell" in claim 81; x) "reconstituting tissues with genetically modified embryonic stem cells" in claim 82; xi) "where the embryonic stem cell has been modified with respect to the histocompatibility antigens present on the stem cell surface" in claim 83; xii) "preparing genetically modified embryonic stem cells" in claim 84; and xiii) "automonously replicating DNA sequences to the genome of a recipient cell" in claim 85.

The amendments to the claims render bases i-vii and ix-x of the rejection moot, however, the amendments are not intended to concede that the specification does not provide written support for the claims prior to amendment. For instance, support for the phrases "expressing a DNA sequence into an animal" and "introducing the transformed donor cell to an organ of an animal, an embryo of an animal or an animal" is found at page 10, line 8; page 11, line 21-page 12, line 9; page 37, lines 7, 12-13, 30, and 32; page 47, lines 18-30; page 50, lines 26-27; page 53, lines 16-22; page 55, line 26-page 56, line 4, and Figure 13C.

Support for "inserted into the vector 3' of the transcription initiation site...with the genome of the cell" is found at page 35, line 5-page 36, line 20 and in Figure 9 (basis viii of the rejection).

Support for "where the embryonic stem cell has been modified with respect to the histocompatibility antigens present on the stem cell surface" is found at page 51, lines 22-31 of the specification (basis xi of the rejection).

Support for "preparing genetically modified ES cells" is found at page 51, line 16 of the specification (basis xii of the rejection), and support for "automonously replicating DNA

sequences to the genome of a recipient cell" is found in Figure 14 and at page 59, line 29-page 61, line 30 of the specification (basis xiii of the rejection).

Hence, withdrawal of the "written description" rejections under § 112(1) is respectfully requested.

The Examiner also rejected claims 77-85 under 35 U.S.C. § 112, first paragraph, as the specification is allegedly not enabling for making transgenic animals other than mice and chickens, for using the method to transduce embryonic stem (ES) cells other than mouse ES cells, and for using the method in gene therapy. This rejection is respectfully traversed.

In particular, the Examiner cites the following to support the position that prior to Applicant's filing non-mouse ES cells did not proliferate in culture and were not capable of making germ cells when introduced to an embryo: Bradley (Biotech., 10:534 (1992)); Seamark (Reprod. Fert. And Devel., 6:653 (1994)); Simkiss (Animals with Novel Genes, Maclean et al. (eds.), pp. 106-137 (1994)); and Mullins et al. (J. Clin. Invest., 98:1557 (1996)). The Examiner also asserts that it was unpredictable how to make transgenic mice without ES cells or transgenic chickens without PGCs. And with regard to gene therapy, the Examiner alleges that vector targeting to desired tissues *in vivo* and the parameters, i.e., promoter, protein, tissue of interest, amount of expression and route of administration to obtain a desired, e.g., therapeutic, effect was unpredictable.

The Examiner is requested to consider that the claims are directed to methods in which cells, e.g., ES cells, having a retrotransposon-based vector are introduced to an animal and the sequences in the vector in those introduced cells expressed in the animal. Thus, it is irrelevant whether the ES cells proliferate in culture or are capable of making germ cells when introduced to an embryo.

With respect to the enablement of ES cells other than mouse ES cells, the Examiner is requested to consider that Thompson (U.S. Patent No. 5,843,780) discloses that ES cells are derived from the embryo and are pluripotent (column 1, lines 21-22), and that pluripotent cell lines have been derived from preimplantation embryos from several domestic and laboratory animal species, citing to Evans et al., Theriogenology, 33, 125 (1990), Notananni et al., J. Reprod. Fertil., 41, 51 (1990); Giler et al., Mol. Reprod. Dev., 36, 130 (1993); Graves, Mol. Reprod. Dev., 36, 424 (1993); Sukoyan et al., Mol. Reprod. Dev., 36, 148 (1993); Sukoyan et al.,

Mol. Reprod. Dev., 33, 418 (1992); and Iannaccone et al., Dev. Biol., 163, 288 (1994) (column 3, lines 39-48), e.g., animals such as rabbit, mink, and rat. Although Thompson questions whether pluripotent cell lines derived from preimplantation embryos from domestic and laboratory animal species are "true" ES cells (column 3, lines 49-56), pluripotent ES-like cells other than mouse ES cells were known to the art prior to Applicant's effective filing date.

The Examiner is also requested to consider that page 654 of Seamark discloses that there are "reports which clearly indicate that pluripotent ES cells can be created for the majority of livestock breeds". Moreover, Vick et al. (Proc. R. Soc. Lond. B, 251, 179 (1993)) (a reference cited by the Examiner to support the proposition that only mouse ES cells and chicken PGCs had been successfully used to make transgenic mice and chickens) disclose that most attempts to isolate and culture inner cell mass (ICM) cells from other species are based on the methods used for the mouse (page 179) and Mullins et al. (a reference cited by the Examiner) disclose that isolated totipotent cells from ICMs have been injected into blastocysts to produce chimeric offspring in both sheep and cattle (page 1558). Further, Mullins et al. (Hypertension, 22, 630 (1993)) state that a number of transgenic species other than mice have been reported, including rat, rabbit, pig, sheep, goat and cow. In addition, Seamark notes that approaches to prepare transgenic livestock species other than those which employ ES cells are available, such as nucleus transfer (citing to a 1986 and a 1987 article) and Wall (Theriogenology, 45, 57 (1996)) (see information disclosure for this application) states that the same procedures useful to prepare transgenic mice are applicable to preparing transgenic farm animals (page 57). Wall also refers to the "striking phenotypes" of transgenic animals having a recombinant growth hormone gene linked to a metallothionine promoter, the enhanced wool production in transgenic sheep which express insulin-like growth factor I, and pigs employed as bioreactors for human hemoglobin (pages 59-60).

Clearly, it was within the skill of the art to prepare transgenic animals other than transgenic mice and chickens.

With regard to the predictably of gene transfer and subsequent expression in animals, the Examiner is requested to consider the following documents. Grunkemeyer et al. (Gene Therapy and Molecular Biology, 6, 91, (2001, a copy is enclosed)) for the first time disclose the use of a lung-specific VL30 retrotransposon to create transgenic mice, via ES cell transgenesis, which

retain type-2 pneumocyte specificity of gene expression in offspring. The technique of rescuing tissue- and factor-specific VL30 transcriptional promoters from VL30 retrotransposon LTRs is disclosed by Staplin et al., (Blood Cells, Molecules, and Diseases, 28, 275, (2002). Staplin et al. (Blood, 101, 1798, (2002), a copy is enclosed) also disclosed sustained long-term gene expression in erythroid progenitor cells using the rescued VL30 LTR promoters. (Cook et al. (Poultry Science, 72, 554 (1993)) (see information disclosure for this application) disclose the use of a retroviral vector to introduce a marker gene under the control of a liver-specific promoter into early Line 11 Leghorn embryos. After hatching, most tissues in the birds contained the marker gene. An ELISA was used to determine the level of expression of the marker gene. The results showed that the marker gene was expressed preferentially in the liver.

Bosselman et al. (U.S. Patent No. 5,162,215) generally disclose the use of a retroviral vector to transfer a nucleic acid sequence to pluripotent stem cells of a chicken embryo. A spleen necrosis virus-derived retroviral vector containing the coding sequence for chicken growth hormone (cGH) was injected beneath and around the blastoderm of unincubated or briefly incubated chicken embryos. The majority of the injected embryos expressed cGH at an elevated level in their sera.

Humans who have inherited the human class I major histocompatibility allele HLA-B27 have a markedly increased risk of developing spondyloarthropathies. Hammer et al. (Cell, 63, 1099 (1990)) introduced the B27 and human beta 2-microglobulin genes into rats by microinjection of DNAs having the genes into fertilized eggs. Rats from one transgenic line displayed a pattern of disease that showed a striking resemblance to the B27-associated human disorder.

A construct having the rabbit beta-casein promoter linked to the genomic human interleukin-2 (hIL2) gene was introduced, via microinjection of the DNA, to fertilized rabbit eggs (Bühler et al., Biotechnology, 2, 140 (1990). The mammary glands of the transgenic rabbits produced milk that contained biologically active hIL2.

Wright et al. (Biotechnology, 9, 830 (1991)) prepared transgenic sheep having the beta-lactoglobulin promoter fused to the genomic human alpha 1-antitrypsin (hα1AT) gene. Biologically active hα1AT was present in the milk of transgenic sheep.

Transgenic goats were obtained by microinjection of DNA into fertilized eggs (Ebert et al., Biotechnology, 9, 835 (1991)). The injected DNA encoded a glycosylation variant of human tissue plasminogen activator (tPA). Milk was obtained from transgenic goats that contained active tPA.

Simons et al. (Nature, 328, 530 (1987)) describe the introduction of the sheep beta-lactoglobulin (BLG) gene into the mouse genome via microinjection of DNA into fertilized eggs. BLG was specifically and abundantly expressed in the mammary gland of transgenic mice during lactation.

Further evidence that the introduction of retroviruses or retroviral vector-transduced cells into an organism can result in expression of the gene which is inserted into the vector is provided by many literature references: Lim et al. (Proc. Natl. Acad. Sci. USA, 86, 8892 (1989)), Flowers et al. (Proc. Natl. Acad. Sci. USA, 87, 2349 (1990)), Garver et al. (Science, 237, 762 (1987)), O'Malley et al. (Hum. Gene Ther., 4, 171 (1993)), Rosenberg (JAMA, 268, 2416 (1992)), Anderson (U.S. Patent No. 5,399,346), Kantoff et al. (J. Exp. Med., 166, 219 (1987)), Hatzoglou et al. (J. Biol. Chem., 265, 17285 (1990)), Nable et al. (Science, 249, 1285 (1990)), Smith et al. (J. Virol., 65, 6365 (1991)), Price et al. (Proc. Natl. Acad. Sci. USA, 84, 156 (1989)), Banjerjee et al. (Stem Cells, 12, 378 (1994)), Vinh et al. (The Journal of Pharmacology and Experimental Therapeutics, 267, 989 (1993)), and Spencer et al. (Blood, 87, 2579 (1996)). Lim et al. disclose that hematopoietic cells transduced with a retroviral vector containing the hADA gene were introduced into mice. hADA was present in peripheral blood samples of 100% of the mice 30 days after transplantation, and in almost 50% of the mice 4 months after transplantation.

Flowers et al. employed retrovirus-mediated gene transfer to introduce the *neo* gene into canine keratinocytes. The transduced keratinocytes were then transplanted into the host donor of the cells. Some of the keratinocytes which were obtained at 27-130 days after transplantation were resistant to G418.

Garver et al. transduced mouse fibroblasts with a retroviral vector encoding human alpha1-antitrypsin and then introduced these cells into 11 mice. Human alpha1-antitrypsin was detected in the serum and on epithelial surface of the lung in 11/11 mice having the transduced fibroblasts.

O'Malley et al. transduced explanted canine follicular cells with a retroviral vector carrying a marker gene. Transduced cells were stained with a vital fluorescent dye and transplanted into the contralateral thyroid lobe of recipient animals. Engraftment of the transduced cells was demonstrated by fluorescence microscopy, and by identification of proviral transcripts and proviral sequences, after transplantation.

Successful treatment of two melanoma patients was reported by Rosenberg. Following *ex vivo* retroviral transduction of autologous tumor-infiltrating lymphocytes (TIL) with a marker gene, transduced cells were transplanted into each of two patients with metastatic melanoma. Analyses of one patient's peripheral blood and several tumor biopsies revealed gene-transduced cells in circulating blood and in tumor deposits, respectively, up to sixty days post-TIL administration. At the time of publication, one patient had a complete regression of all subcutaneous, oral and lung metastases (2 years, 10 months). Similar results were observed with a second patient.

Anderson et al. generally disclose a method that includes *ex vivo* retroviral transduction of human cells with DNA encoding a therapeutic protein and administration of the transduced cells to a human. In Example 5, it is disclosed that a patient having adenosine deaminase (ADA) deficiency received T lymphocytes that had been transduced *ex vivo* with a retroviral vector encoding ADA. Lymphocyte levels of ADA were increased up to 180 days post-administration.

Kantoff et al. transduced primate hematopoietic cells with a retroviral vector containing the *neo* and human adenosine deaminase (hADA) genes. The transduced cells were then introduced into five monkeys. Hematopoietic cells isolated from 4/5 monkeys expressed hADA. The hematopoietic cells of two of the monkeys expressed the *neo* gene 169 days, and expressed ADA 120 days, post-transplantation.

Hatzoglou et al. infected the liver of fetal rats with a retroviral vector containing a predominantly liver-specific promoter linked to a marker gene. 18/79 of the live-born offspring had detectable levels of the marker gene at two months of age. Hatzoglou et al. also infected the liver of adult rats with the vector. 4/40 adults rats expressed detectable amounts of the marker gene three weeks after infection.

Nabel et al. employed a catheter to introduce recombinant retrovirus containing the β-galactosidase gene to swine arteries. β-galactosidase activity was detectable in arterial

endothelial cells exposed to the virus. Price et al. detected β-galactosidase activity in rat retinal cells after rat retinas were exposed to β-galactosidase gene-containing retrovirus.

Banjerjee et al. review the use of retroviral vectors to transfer and express drug resistance genes in animals. Banjerjee et al. describe the use of retroviral vectors to transfer a drug resistance gene to murine bone marrow cells and the subsequent transplantation of those cells to mice. This led to the successful protection of the recipient marrow from MTX toxicity (page 381). The actual infection of an early stem cell was further demonstrated by serial transplantation of the marrow to secondary recipients who were also shown to be protected similarly (page 381). Banjerjee et al. also teach that work on *in vivo* gene transfer of the multidrug resistance gene (MDR) in a retroviral vector demonstrated that animals receiving the MDR gene expressed the gene in leukocytes (page 382).

Vinh et al. teach that "[t]he use of retroviral vectors has proven to be an effective method of introducing new gene sequences into hematopoietic tissue of animals" (page 990) and report the use of retroviral vectors to efficiently introduce and to functionally express a gene in hematopoietic cells that were introduced into recipient mice (page 990).

Spencer et al. report the attenuation of myelosuppression in mice transplanted with bone marrow cells transduced with a retroviral vector containing a foreign gene designed to provide resistance to myelosuppression following trimetrexate treatment (bridging sentence from pages 2579-2580).

Furthermore, Anderson (Science, 256, 808 (1992)) (information disclosure, this application) reviews 20 approved clinical trials (Table 1) and further describe several examples where gene therapy protocols resulted in beneficial effects. In addition, Anderson describes results from ADA gene therapy and concludes that "[t]he data from the protocol indicate that this therapy was clinically useful" (emphasis added) (page 256, column 1, paragraph 1). The Examiner is also requested to consider VandenDriessche et al. (Curr. Gene Therapy, 3, 501 (2003)) which discloses that nearly 2,000 patients have been enrolled in gene therapy clinical trials with retroviral vectors. Clearly, clinical trials are not approved based on methodologies that are wholly unpredictable.

Applicant's Representatives respectfully submit that these references are sufficient to rebut the Examiner's assertion that gene transfer and expression *in vivo* is unpredictable. Indeed,

as is illustrated by the documents submitted herewith, the use of vectors, including retroviral vectors, to transfer and express foreign genes *in vivo* was routine and predictable as of the effective filing date of the present application. Moreover, the above-cited documents provide evidence that gene therapy can produce beneficial effects.

The Examiner cites to Miller et al. (FASEB J., 9, 190 (1995)), Crystal (Science, 270, 404 (1995)), Deonarain (Exp. Op. Ther. Pat., 8, 53 (1998)) and Verma (Nature, 389, 239 (1997)) to support the proposition that vector targeting to desired tissues *in vivo* and gene therapy is unpredictable.

Crystal summarizes 28 studies of human gene transfer (Table 1) and teaches that "retroviral vector DNA or marker gene-derived mRNA or both have been observed in cells collected after periods ranging from several weeks to 36 months after administration" (page 405, column 3, paragraph 3). Moreover, Crystal summarizes "(a)lthough gene transfer has not been shown in all recipients, most studies have shown that genes can be transferred to humans whether the strategy is *ex vivo* or *in vivo*, and that all vector types function as intended. Taken together, the evidence is overwhelming, with successful gene transfer having been demonstrated in 28 *ex vivo* and 10 *in vivo* studies." (emphasis added; page 405, column 3, paragraph 2). Further, Schmidt et al. (Hepato-Gastroenterology, 44, 1013 (1997)) review a somatic gene therapy trial in which autologous hepatocytes were transduced *ex vivo* with a retroviral vector containing the human LDL-receptor gene. The transduced hepatocytes were administered to patients who lack this gene, resulting in a stable reduction of LDL-cholesterol of up to 20% in those patients (page 1017, column 1 final paragraph through column 2, first paragraph).

Miller et al. describe the use of genetically engineered viral glycoproteins to confer modified, specific binding affinity as "a promising approach" (page 191). Moreover, Miller et al. refer to three reports of modifications to a retroviral glycoprotein, two of which led to an altered cell tropism (page 192). Miller et al. also describe the use of retroviral ligand conjugates to target virus to a particular cell. They disclose that of three reports directed to this type of targeting approach, two were successful and the third showed partial success. Although Miller et al. and Verma et al. refer to the inefficiency of gene delivery, there is no disclosure or suggestion in any of the above-referenced articles that gene transfer and expression cannot be routinely achieved.

With regard to claims 81 and 85, the Examiner asserts that because the specification does not describe a method of producing a gene capable of homologous recombination using a retrotransposon-based vector (claim 81) or a method of delivering an autonomously replicating sequence to a cell, it cannot be determined whether those methods are enabled. Figure 9 and page 35, line 5-page 36, line 20 describe a LTR containing vector with sequences flanked by targeting (homologous) sequences. Once such a vector is introduced to a cell and reverse transcribed, it is a homologous targeting vector. Figure 14 and page 59, line 26-page 61, line 30 disclose the introduction of autonomously replicating sequences, e.g., transposons or other excisable elements, to a LTR based vector, e.g., retrotransposon-based vector.

It is respectfully submitted that § 112, first paragraph, requires no more than a disclosure sufficient to enable one skilled in the art to carry out the invention commensurate with the scope of the claims and this requirement has clearly been met.

Accordingly, withdrawal of the enablement rejections under § 112(1) is respectfully requested.

*The 35 U.S.C. § 112, Second Paragraph, Rejections*

The Examiner rejected claims 77-85 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. This rejection, as it may be maintained with respect to the pending claims, is respectfully traversed.

In particular, the Examiner asserts that a) claims 77 and 80 are indefinite because DNA sequences are not expressed "into" an animal; b) claims 77, 80 and 85 are indefinite because the metes and bounds of "donor cell" are unclear, as it is unclear if the cells are packaging cells or infected with a virus made by a packaging cell line; c) the phrase "to yield a transformed donor cell" in step a) of claims 77, 81 and 85 is unclear; d) use of the word "linked" in step a) of claims 77, 80-82, and 84-85 is confusing, as it cannot be determined if the word is intended to mean elements i-vii are connected in the order shown or have some particular structure beyond merely being in the vector; e) claims 77, 80-82, 84, and 85 are indefinite because "from step (d)(1)" does not have antecedent basis; f) the phrase "introducing the transformed donor cell to" is confusing in claims 77, 80, and 82; g) claims 77 and 80 are indefinite because step c) is not commensurate

in scope with the preamble of the claim; h) claim 78 is indefinite because an embryo is not a cell; i) claim 80 is indefinite because the phrase "capable of packaging nucleic acid molecules into a virion to yield a transformed donor cell" is confusing; j) claim 81 is indefinite because step b) is not commensurate in scope with the preamble of the claim; k) the phrase "which is capable of homologous recombination with the genome of the cell" in claim 81 is unclear; l) the phrase "to yield a transformed embryonic stem cell" in step a) of claims 82 and 84 is unclear; m) the term "donor cell" in claim 82 lacks antecedent basis; n) claims 82 and 84 are confusing because a virion must be used to infect embryonic stem cells; o) claim 82 is indefinite because step c) is not commensurate in scope with the preamble of the claim; p) claim 82 is indefinite because the metes and bounds of "reconstituted" cannot be determined; q) claim 83 is indefinite as the structural or functional modification "with respect to the histocompatability antigens present on the stem cell surface" cannot be envisioned; r) claim 84 is indefinite because step b) is not commensurate in scope with the preamble of the claim; s) the metes and bounds of "autonomously replicating DNA sequences" in claim 85 is unclear; t) use of "recipient cell" in the preamble of claim 85 and "donor cell" in step a) of claim 85 is confusing; and u) claim 85 is indefinite because step b) is not commensurate in scope with the preamble of the claim.

The amendments to the claims obviate bases a, c-m, o-p, r, and t-u of the rejection.

With regard to bases b and n of the rejection, cells including embryonic stem cells or packaging cells, which are infected or transfected with a retrotransposon-based vector may be introduced to an animal, which can yield an animal with cells that express sequences in the vector.

As discussed at page 51, lines 22-27 of the specification, modifications to histocompatibility antigens were known at the time of Applicant's filing (basis q of the rejection). Moreover, autonomously replicating sequences (ARS), such as transposons and mouse minute virus (Figure 14 and page 59, line 29-page 61, line 30), were also known to the art prior to Applicant's filing (basis of the rejection). Thus, one of ordinary skill in the art in the absence of Applicant's specification, or alternatively in possession of Applicant's specification, would be apprised of the metes and bounds of "modifications to histocompatibility antigens" and "autonomously replicating sequences".

Therefore, withdrawal of the § 112(2) rejections is appropriate and is respectfully requested.

## CONCLUSION

Applicant respectfully submits that the claims are in condition for allowance, and notification to that effect is earnestly requested. The Examiner is specifically requested to telephone Applicant at (402) 472-6530 to facilitate prosecution of this application by the inventor, *pro se*.

## CONDITIONAL REQUEST FOR CONSTRUCTIVE ASSISTANCE

Applicant has amended the specification and claims of this application so that they are proper, definite, and define novel structure which is also unobvious. If, for any reason this application is not believed to be in full condition for allowance, applicant respectfully requests the constructive assistance and suggestions of the Examiner pursuant to M.P.E.P. § 2173.02 and § 707.07(j) in order that the undersigned can place this application in allowable condition as soon as possible and without the need for further proceedings.

Respectfully submitted,

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Name

Signature

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**Article Title: BVL-1 like VL30 Promoter Sustains Long Term Expression in Erythroid Progenitor Cells**

Running Header Title: VL30 Promoter Directs Sustained Erythroid Activity

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## ABSTRACT

Congenital blood disorders are common, and yet clinically challenging globin disorders. Gene therapy continues to serve as a potential therapeutic method to treat these disorders. While tremendous advances have been made *in vivo*, gene delivery protocols and vector prototypes still require optimization. Alternative *cis* acting promoter elements derived from VL30 retroelements have been effective in expressing tissue-specific transgene expression, *in vivo*, in non-erythroid cells. VL30 promoter elements were isolated from ELM-I-1 erythroid progenitor cells upon erythropoietin (epo) treatment. These promoters were inserted into a VL30-derived expression vector and reintroduced into the ELM-I-1 cells. β-galactosidase reporter gene activity from the ELM 5 clone, a BVL-1 like VL30 promoter, was capable of expressing sustained levels of the transgene expression over a sixteen week assay period. These findings delineate the potential utility of these retroelement promoters as transcriptionally active, erythroid specific, LTR components, for current globin vector constructs.

## INTRODUCTION

Retroviral and lentiviral model systems are close to being utilized for hemoglobinopathy human gene therapy trials (1). Incorporation of *cis* acting regulatory components of the globin gene, including the Locus Control Region (LCR) initially caused aberrant RNA processing. Modifications have allowed significant levels of recombinant globin gene expression to be achieved (2, 3, 4). Retroviral vectors utilizing the promoter from the red cell membrane protein ankyrin have shown position-independent, copy number dependent expression of the gamma globin gene, *in vivo* (5). However, significant obstacles still face the contemporary constructs, including silencing regions within the vectors' Long Terminal Repeat (LTR) regions, and position effect variegation (PEV) (6). Removal of silencing regions within the vectors' promoter (U3), repeat (R), and primer binding site (PBS) and insertion of insulators has not entirely prevented transcriptional silencing (7) and PEV (6), respectively. Optimization of specific globin vector designs is requisite to allow for therapeutically beneficial and sustainable globin gene expression.

A variation on virally derived LTR vector components is the use of retrotransposon-based VL30 U3 promoter sequences. Mouse VL30s include over one hundred different promoter variations within the endogenous genome, have limited sequence homology to murine retroviruses (8), and are activated by transformation (9, 10), growth factors (11, 12), steroids (13), and cytokines (14). VL30 promoters have been characterized for tissue specific expression *in vitro* (15, 16) and *in vivo* (17). Hematopoietic VL30 elements (14), and specialized erythropoietin (epo)-responsive erythroid progenitor cell lines have been

probed (20). However, these clones have not been assessed in the context other than their native configuration. Therefore, VL30 promoter elements within a heterologous construct were reintroduced into erythroid progenitor cells. These expression assays determined that the ELM 5 (BVL-1 like) VL30 clone provided sustained reporter gene expression in erythroid progenitor cells, *in vitro*.

## METHODS

***VL30 Promoter Selections.*** VL30 promoters were isolated from MEL 585S (18) and ELM-I-1 (19) murine erythroleukemic cell lines. Ribonuclease protection assays (RPA) were performed to confirm the epo-inducibility of the VL30 promoter clones in the epo responsive ELM-I-1 cell line (20).

***Electroporation into ELM-I-1 Erythroid Progenitor Cells.*** Linearized constructs with the novel promoters, ELM5 and MEL/ELM, and control promoter, NVL3, within the LTRs of the VL30-derived VLIBAG cassette (17; ), and the retroviral vector RVBAG, were electroporated into the ELM cells (10 – 20 x $10^6$), using a voltage of 300 V, resistance of 13 Ω, and a capacitance of 200 F. Mass cell cultures of transfected cells were grown under selective G418 growth conditions (0.8 mg/ml).

***X-Gal Staining of VLIBAG Transfected Cells.*** Transfected cells were stained with Xgal (X-Galactopyranoside 1mg/ml in N, N,-dimethyl formamide) along with Xgal staining solution (20 mM $K_3Fe(CN)6$; 20 mM $K_4 Fe(CN)_6$ $3H_20$; 1.5 mM $MgCl_2$ in PBS, pH 7.4) and incubated for 20 hours at 37° C (21).

***ONPG Hydrolysis of Cell Lysates from Stably Transfected ELM Cells.*** Transfected ELM cells (2.5 – 4 x $10^5$ cells/mL) were grown for 3 days in uninduced (0.55 unit/mL) and epo induced (10 unit/mL) growth conditions in T75 Falcon flasks for 3 days. Cell lysates were monitored through duplicate assays, performed in triplicate. Using the β-Gal Assay Kit (Invitrogen™), the specific activity of the cell lysates (nmoles of ONPG hydrolyzed per mg of total protein) from each vector construct was calculated, and normalized to the total protein concentration (Pierce). The uninduced VLIBAG vector's expression was defined from each time point as one nmole of ONPG hydrolyzed per milligram of protein in 30 minutes at 37° C.

***Statistical Analysis of Expression Assays.*** Raw data from each assay was assessed through a paired correlated groups Student t-test, using the InStat program (GraphPad Software, Inc.-Version 3.01). Probability (P) values that were less than 0.05 were considered significant.

## RESULTS and DISCUSSION

***VL30 Promoter Isolations.*** Degenerate VL30 specific primers were employed to reverse transcribe (RT) a single-stranded 3' U3 promoter complementary DNA (cDNA), (20), Figure 1A. Polymerase chain reaction (PCR) was utilized to synthesize and amplify double-stranded cDNA clones from MEL and ELM-I-1 cell RNA. VL30 promoter clones were identified, and classified (20). The MEL/ELM clone was conserved between both erythroid progenitor cell lines and was grouped with members of the VL30 subgroup IV

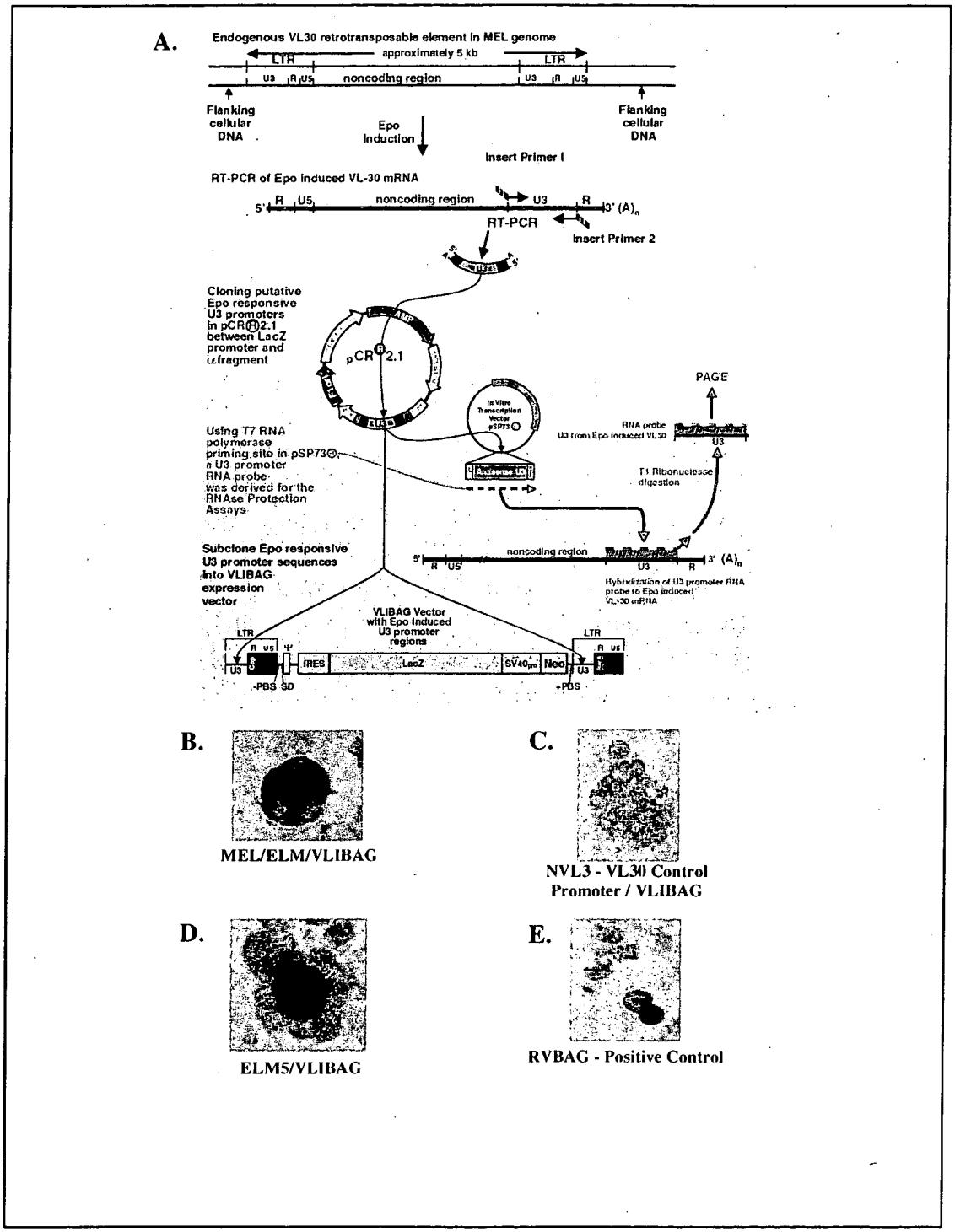


**Figure 1. Endogenous ELM 5 and MEL/ELM Promoters Expessing Reporter Gene Activity in Transfected ELM Cells**. ELM VL30 promoters were isolated, confirmed, and quantified for their epo responsiveness (20), and cloned into the LTRs of the expression vector, VLIBAG (A). Mass cultures of ELM transfectants carrying the endogenously expressed promoters MEL/ELM (B) and ELM 5 (C) within the heterologous cassette, VLIBAG, were histochemically stained with X-Gal after two weeks of selective growth conditions. The unexpressive negative control transfectant, VLIBAG with the NVL3 promoter is shown (D) as well as the positive control RVBAG retroviral vector (E).

(21). The other five promoters were categorized in subgroup III (21). Clone ELM 5 showed homology (97%) to a previously identified VL30 element, BVL-1 (GenBank Number X17124) (14, 22), and contained a putative erythropoietin activated Jak2/STAT5 recognition site (23). Sequences of the ELM 5 and MEL/ELM clones encoded three GATA-1 sites (20) and two B10 Ras-responsive elements (12). Confirmatory ribonuclease protection of the various VL30 promoters verified the expression of the isolated VL30 promoters (20).

The VL30 promoters MEL/ELM and ELM 5 were cloned into the promoter regions of the VL30-derived expression vector, VLIBAG (GenBank Number AF062997), for electroporation into the ELM cells (Figure 1A). A fibroblast specific VL30 promoter, NVL3 (17, 24), as well as the positive control, the retroviral vector RVBAG (25), were also electroporated into the ELM cells. Transfected cells were grown in selective media and initially stained with X-gal after two weeks post transfection, to confirm the constructs' reporter gene expression (Figures 1B-E). In contrast to the control NVL3 promoter, MEL/ELM and ELM 5 constructs showed insignificant reporter gene expression levels in transformed fibroblast cell lines (data not shown).

***Transfected ELM Cell ONPG Hydrolysis Assays.*** The transfected ELM cells were grown simultaneously under uninduced (0.5 units/mL epo) and epo-induced (10 units/mL epo) growth conditions. The specific activity for each of the transfected cells was monitored over two week time intervals for sixteen weeks.

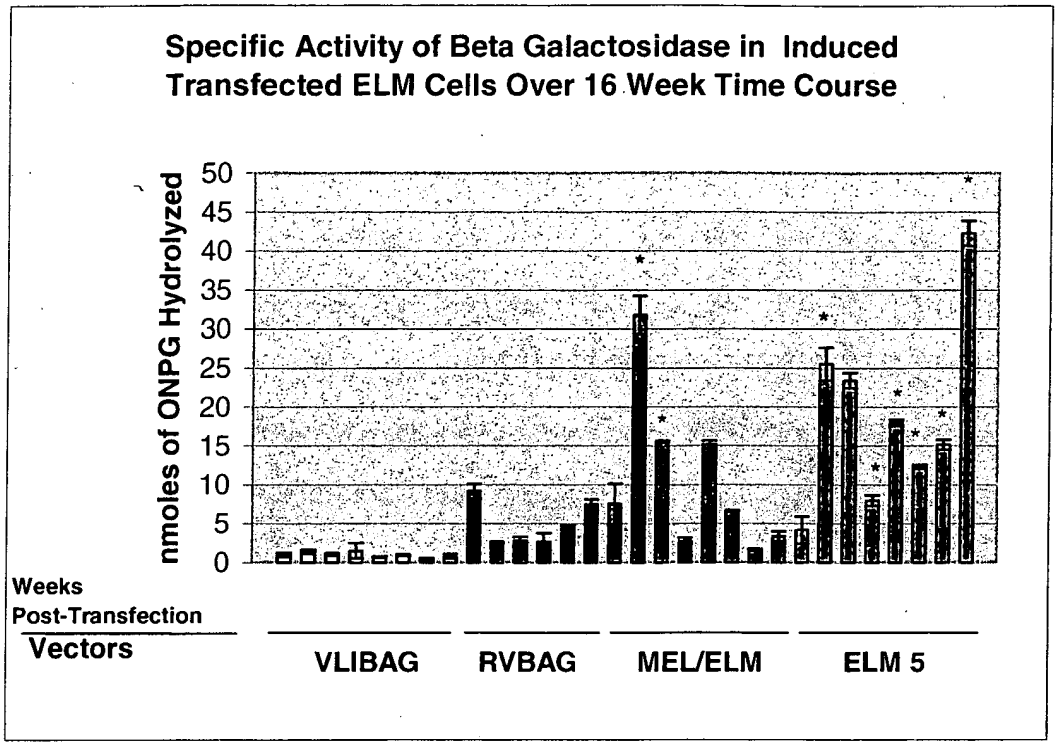


**Figure 2. Beta (β)-Galactosidase Specific Activity of BVL-1 like, ELM 5 Promoter Shows Sustained Expression Patterns in Transfected ELM Clones.** The VL30 promoters ELM 5, MEL/ELM, negative control NVL3 promoter within VLIBAG and the positive retroviral control vector, RVBAG were transfected into ELM-I-1 cells and monitored for β-galactosidase reporter gene activity, through ONPG hydrolysis assays over a 16 week time course. β-galactosidase reporter gene activity was monitored in cell lysates from all four constructs in epo induced (10 Unit/mL epo) growth conditions. The transfected BVL-1 like ELM 5 vector showed sustained β-galactosidase activity and modest epo induction ($P < 0.05$, $n = 6$) on weeks 4, 8, 10, 12, 14, and 16 (*).

The NVL3 control promoter in the VLIBAG construct showed little activity throughout the time course assay (Figure 2). The specific activity of the positive control vector, RVBAG was greatest after two weeks in culture and showed minimal expression by the twelfth week (Figure 2). ELM cells transfected with the novel MEL/ELM promoter showed significant β-galactosidase expression during the $4^{th}$ and $6^{th}$ weeks of post transfection. However, this construct also expressed minimal activity by the 16th week (Figure 2). ELM 5 transfected cells showed sustained β-galactosidase activity (Figure 2), averaging 18.64 nmoles of hydrolyzed ONPG in epo induced growth

conditions. Six of the eight time points: week 4 (P = 0.0441), week 8 (P = 0.019), week 10 (P = 0.0086), week 12 (P = 0.0007), week 14 (P = 0.049), and week 16 (P = 0.037) reflected a significant enhancement of reporter gene activity, Figure 2. Even though the epo induction was modest, 1.2 to 1.7 fold, (data not shown), the ELM 5 promoter within the VL30 vector was able to direct sustained expression throughout the sixteen week time course in either growth conditions.

In summary, erythroid specific VL30 promoters were isolated, confirmed for their apparent epo responsive behavior (20), and cloned into an expression vector, VLIBAG. Reintroduction of the novel constructs into the ELM cells revealed that the ELM 5 (BVL-1 like) promoter was able to express itself consistently during the sixteen week assay period. This promoter was able to direct sustainable expression levels throughout the time course assay, and continued to express itself past sixteen weeks in culture. The incorporation of this promoter within the flanking LTR regions of contemporary vectors may allow for sustained therapeutic levels of transgenic globin expression.

## ACKNOWLEDGEMENTS

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## REFERENCES


1. Wilson, J.F. Murine gene therapy corrects symptoms of sickle cell disease. The Scientist. 2002; 16; 36.

2. May, C., Rivella, S., Callegari, J., Heller, G., Gaensler, K.M.L., Luzzatto, L., and Sadelain, M. Therapeutic haemoglobin synthesis in β-globin. Nature. 2000; 406: 82-86.

3. Pawliuk, R., Westerman, K.A., Fabry, M.E., Payen, E., Tighe, R., Bouhassira, E.E., Acharya, S.A., Ellis, J., London, I.M., Eaves, C. J., Humphries, K., Beuzard, Y., Nagel, R.L., Leboulch, P. Correction of sickle cell disease in transgenic mouse models by gene therapy. Nature. 2001; 294: 2368-2371.

4. Tisdale, J., Sadelain, M. Toward gene therapy for disorders of globin synthesis. Semin. Hematol. 2001; 38: 382-392.

5. Sabatino, D.E., Seidel, N.E., Aviles-Mendoza, G.J., Cline, A.P., Anderson, S.M., Gallagher, P.G., Bodine, D.M. Long-term expression of γ-globin mRNA in mouse erythrocytes from retrovirus vectors containing the human γ-globin gene fused to the ankyrin-1 promoter. Proc Natl Acad Sci USA. 2000; 97:13294-13299.

6. Rivella, S., Callegari, J.A., May, C., Tan, C.W., Sadelain, M. The cHS4 Insulator increases the probability of retroviral expression at random chromosomal integration sites. J Virol. 2000; 74: 4679-4687.

7. Osborne, C.S., Pasceri, P., Singal, R., Sukonnik, T., Ginder, G.D., Ellis, J. Amelioration of retroviral vector silencing in Locus Control Region β-globin-

transgenic mice and transduced F9 embryonic cells. J Virol. 1999; 73: 5490-5496.

8. Bosze, Z., Thiesen, H.J., Charnay, P. A transcriptional enhancer with specificity for erythroid cells is located in the long terminal repeat of the Friend murine leukemia virus. EMBO J. 1986; 5: 1615-1623.

9. French, N.S., Norton, J.D. Structure and functional properties of mouse VL30 retrotransposons. Biochem Biophys Acta. 1997; 1352: 33-47.

10. Wolgamot, G., Miller, A.D. Replication of *Mus dunni* endogenous retrovirus depends on promoter activation followed by enhancer multimerization. J Virol. 1999; 73: 9803-9809.

11. Foster, D.N. Schmidt, L.J., Hodgson, C.P. et al. Polyadenylated RNA complementary to a mouse retro-virus like multigene family is rapidly and specifically induced by epidermal growth factor stimulation of quiescent cells. Proc Natl Acad Sci USA. 1982; 79: 7317-7321.

12. Nilsson, M., Ford, J., Bohm, S., Toftgard, R. Characterization of a nuclear factor that binds juxtaposed with ATF3/Jun on a composite response element specifically mediating induced transcription in response to an epidermal growth factor/Ras/Raf signaling pathway. Cell Growth Differ. 1997; 8: 913-920.

13. Harrigan, M.T., Baughman, G., Campbell, N.F., Bourgeois, S. Isolation and characterization of glucocorticoid and cAMP-induced genes in T lymphocytes. Mol Cell Biol. 1989; 9: 3438-3446.

14. Park, D.J., Lim, R.W., Kim, H.D. Rapid induction of mouse virus-like (VL30) element transcripts by erythropoietin in murine erythroid progenitor cells. Blood. 1993; 82: 77-83.

15. Cook, R.F., Cook, S.J., Hodgson, C.P. Retrotransposon gene engineering. Bio Technology. 1991; 9: 748-751.

16. Chakraborty, A.K., Zink, M.A., Hodgson, C.P. Expression of VL30 vector in human cells that are targets for gene therapy. Biochem Biophys Res Comm. 1995; 209: 677-683.

17. Grunkemeyer, J.A., Hodgson, C.P., Cosgrove, D. Sustained tissue-specific transgene expression from a VL30 retrotransposon-derived vector in vivo. Gene Ther Mol Biol. 2001; 6: 91-99.

18. Baron, M.H., Maniatis, T. Rapid reprogramming of globin gene expression in transient heterokaryons. Cell. 1986; 46: 591-602.

19. Iwatuski, K., Endo, T., Misawa, H., Yokouchi, M., Matsumoto, A., Motoaki, O., Mori, K.J., Yoshimura, A. STAT5 activation correlates with erythropoietin receptor-mediated erythroid differentiation of an erythroleukemia cell line. J of Vir. 1997; 272: 8149-8152.

20. Staplin, W.R., Hodgson, C.P., Knezetic, J.A. Conserved, erythropoietin-responsive promoters isolated from erythroid progenitor cells. Blood Cell, Mol, and Dis. 2002; 28: 275-282.

21. Ausubel, F.M.,ed., Brent, R., Kingston, R.E., Moore, D.D., Seideman, J.G., Smith, J.A., Struhl, K. Current Protocols in Molecular Biology. New York, N.Y: John Wiley & Sons, Inc.; 1997.

22. Hodgson, C.P., Fisk, R.Z., Arora, P., Chotani, M. The nucleotide sequence of a mouse virus-like (VL30) retrotransposon BVL-1. Nucleic Acids Res. 1990;18: 673.

23. Darnell, J.E. STATs and gene regulation. Science. 1997; 277:1630-1635.

24. Adams, S.E., Rathjen, P.D. Stanway, C.A. et al. Complete nucleotide sequence of a mouse VL30 retroelement. Mol Cell Biol. 1988; 8: 2989-2998.

25. Price, J., Turner, D., Cepko, C. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc Natl Acad Sci USA. 1987; 84: 156-160.

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# Conserved, Erythropoietin-Responsive VL30 Promoters Isolated from Erythroid Progenitor Cells

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**ABSTRACT:** Virus-like 30S (VL30) elements are endogenous retro-elements of the mouse retrotransposon family. These elements are transcriptionally responsive in a temporal and tissue-specific manner due to the U3 promoter region of the elements' long terminal repeat (LTR). We have analyzed VL30 promoters from erythroid progenitor cell lines (MEL 585S and ELM-I-1) that contrasted in their response to erythropoietin (epo). Through RT-PCR-generated cDNAs, VL30 promoters were identified and showed homology to the third and fourth U3 subgroups, with GATA-1, Jak2/STAT5, and B10 RRE sites. One clone (ELM5) showed 97% homology to BVL-1, a putative epo-responsive VL30 element. In addition, a novel U3 promoter (MEL/ELM CONSTIT) showed complete sequence homology between both cell lines. Ribonuclease protection confirmed that epo-induced VL30 promoters were activated in ELM-I-1 cells, whereas the conserved VL30 MEL-ELM CONSTIT VL30 promoter showed no enhanced expression in the epo-unresponsive MEL cells. Identification of these U3 promoters suggests that VL30s are conserved and can be transcriptionally activated in an epo-specific manner. 

*Key Words:* VL30 elements; ELM-I-1 erythroid progenitor cells; erythropoietin; BVL-1.

## INTRODUCTION

VL30s "virus-like 30S" retro-elements are one family of endogenous, noninfectious, mouse retrotransposons that are mobile and transcriptionally active in the murine genome (1). VL30s have long terminal repeats, LTRs, which flank a single 5- to 10-kilobase transcriptional unit. The unique 3′ (U3), promoter regions of the VL30s' LTR activates the duplicative retrotransposition process and classifies the VL30 elements into five subgroups (2). The first and fourth VL30 U3 subgroups are transcriptionally active variants of NVL1/2 and NVL3, respectively (1). Subgroup II represents genomic library isolates, subgroup III is transcriptionally active and distinct from the NVL subsets (1), and MDEV (*Mus dunni* endogenous virus) represents a fifth VL30 family (2). These five subgroups have been categorized based on specific cell type distinctive expression. Transcriptionally active U3 promoters are activated in a temporal and tissue specific manner, *in vivo* (1, 3, 4) by various mitogenic (5–8) and extracellular factors (6, 9–13). Hematopoietic VL30 elements were previously isolated from erythroid progenitor cells from the spleens of Friend virus transformed BALB/c mice and showed evidence of epo responsive expression (14). However, the erythroid specific promoter elements, which seemed similar to the BVL-1 VL30 promoter (18) were not further characterized.

To elucidate epo responsive VL30s, endogenous to erythroid progenitor genomes, our laboratory has probed two erythroid progenitor cell lines, MEL 585S and ELM-I-1, for erythropoietin (epo)-induced VL30 retroelements. MEL 585S (15) is epo-unresponsive (16), while ELM-I-1 (17) is epo-responsive. Interestingly, these two cell lines harbor an identical erythroid specific VL30 promoter sequence in their genomes. In

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addition, the ELM-I-1 cells contained an epo responsive VL30 element.

## MATERIALS AND METHODS

### *Induction of VL30 Elements from the Erythroid Progenitor Cells*

Mouse erythroleukemic cell lines, MEL 585S (15), and ELM-I-1 (17) were maintained in Dulbecco's modified Eagle's media (DMEM, high glucose, Life Technologies), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies) and 1× antibiotic (penicillin/streptomycin, Life Technologies). ELM-I-1 cells required 0.5 units/mL of erythropoietin (epo). MEL cells ($6 \times 10^6$ cells/mL) were harvested from the following growth conditions: 2% DMSO (Mallinckrodt) and 12% FBS for 3 to 4 days (15); cycloheximide (Sigma) at 10 mg/mL for 2 h; and epo (2 units/mL) for 2 h (14). ELM-I-1 cells were harvested from the following epo exposures: 0, 0.5, and 10 units/mL for 3 days (17).

### *Reverse Transcription-Polymerase Chain Reaction of VL30 Promoters from Erythropoietin-Induced Erythroid Cells*

Total RNA from the harvested cells was isolated using RNAzolB reagent (Tel-Test, Inc., Friendswood, TX). RNA (1 $\mu$g) from each of the respective growth conditions was treated with deoxyribonuclease I (Life Technologies, Grand Island, NY). Reverse transcription (RT) followed by polymerase chain reaction (PCR) was performed according to manufacturer's protocols (Perkin–Elmer, Foster City, CA). The antisense primer (5′-CGCATCTTTTAATTAACTGGAGAGAATTTTTCACAGGCTTATATAGGAAA-3′; 0.75 $\mu$M) was used for the RT reaction. The U3 promoter cDNA products were PCR amplified with the sense primer (5′-CGATCTTATTAATTAACTGGAGTTTTGAGCCCAACCCCTCCCATC-3′). RT-PCR products and their controls were electrophoresed on 1.5% agarose gels (40 mM Tris–acetate, 1 mM EDTA, 0.5 $\mu$g/mL ethidium bromide).

### *Cloning of Candidate Epo-Responsive VL30 Promoter Elements*

Gel-purified U3 promoter products were cloned into TOPO pCR2.1 vectors (Invitrogen, Carlsbad, CA), and transformed into *Escherichia coli* SCS110-competent cells (Stratagene, La Jolla, CA). Sequences were discerned from a Perkin–Elmer Applied Biosystems 373 fluorescent sequencer and compared through the GrowTree Program (Genetic Computer Group, Madison, WI).

### *Ribonuclease Protection Assays of Endogenous Epo-Responsive VL30 Promoter Elements*

Ribonuclease protection assays (RPA) were performed using Maxiscript and RPAIII hybridization components and protocols (Ambion, Austin, TX). A 151-base antisense probe (Fig. 2, boxed region) was cloned into the *Xho*I site of pSP73 (Promega, Madison, WI). The vector was linearized with *Pvu*II, gel purified, and T7 RNA polymerase synthesized a 177-base anti-sense [$\alpha$-$^{32}$P]CTP-labeled probe (Amersham–Pharmacia Biotech, Piscataway, NJ). A control 304 base pTRI-Actin probe (Ambion) was also synthesized. The VL30 and control probes with their respective specific activities, $1.2 \times 10^5$ cpm and $8 \times 10^4$ cpm, were coprecipitated with uninduced (0.5 units/mL) and epo induced (10 units/mL) total RNA (5, 10, and 15 $\mu$g) from untreated and epo treated (10 units/mL) MEL cells for 2-h and 3-day durations, and ELM-I-1 cells for 3 days. Diluted Rnase T1 (1:75) was added to hybridized RPA products at 37°C for 30 min. [$\alpha$-$^{32}$P]CTP-labeled RNA Century Markers (Ambion) and [$\gamma$-$^{32}$P]ATP-labeled *Msp*I-digested pBR322 (NEBiolabs) (0.5 $\mu$g) indicated molecular sizes. Samples ($n = 5$) were fractionated on a 5% polyacrylamide gel, quantified on a GS-525 Molecular Imager System (Bio-Rad), and analyzed by a paired Student $t$ test (GraphPad Software, Inc., version 3.01).

## RESULTS

### *Reverse Transcription-Polymerase Chain Reaction of Epo-Induced VL30 Promoters*

RT-PCR was utilized to initially detect VL30 U3 promoter elements within the epo treated

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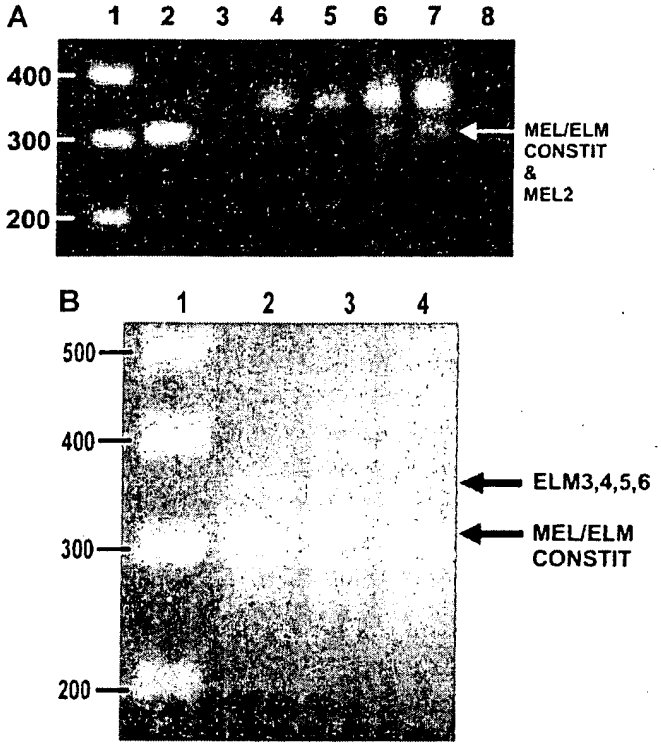


**FIG. 1.** Electrophoresis of MEL 585S and ELM-I-1 VL30 RT-PCR products. (A and B) VL30 RNA U3 promoters were reverse transcribed and amplified from uninduced and epo-induced erythroid progenitor cell lines MEL 585S (A) and ELM-I-1 (B) using VL30 specific U3 primers. Lanes A1 and B1, 100-base-pair (bp) ladder. Lane A2, RT-PCR of the positive control, pAW109 RNA; lane A3, negative control, without RNA; lane A4, uninduced RNA sample; lanes A5–A7; show promoters from treated RNA samples; lane A5, DMSO treatment; lane A6, cycloheximide treatment; lane A7, epo treatment. VL30 promoters, MEL/ELM CONSTIT and MEL2, were isolated from the 270- to 310-bp epo-treated cDNA pool (white arrow). Lane A8, DNAsed RNA control without RT. (B) ELM-I-1 VL30 RT-PCR products. Lane B2, 0 units/ml epo treatment; lane B3, 0.5 units/ml epo treatment; lane B4, 10 units/ml epo treatment with promoters indicated. ELM 3, 4, 5, and 6 were isolated from the epo-treated 350-bp VL30 cDNA pool (lane B4, black arrow). The U3 promoter, MEL/ELM CONSTIT, shown at 305 bp, was isolated independently from the three growth conditions (lanes B2–B4, black arrow).

MEL 585S and ELM-I-1 erythroid progenitor cell lines. The 3′ U3 promoters within the treated total RNA pools (Figs. 1A and 1B) showed a range of cDNA products (350–300 bp). Figure 1A shows MEL cell VL30 U3 cDNA products from DMSO and cycloheximide treated RNA (Fig. 1A, lanes 5 and 6) as well as from the epo treated RNA (Fig. 1A, lane 7). MEL 585S cells have been characterized as being unresponsive to epo (15). Yet, VL30 elements that may have been activated in the lower molecular weight, epo treated VL30 U3 promoter pool (270–310 bp, white arrow) were further characterized by cloning into the TA cloning vector pCR2.1 (labeled as MEL/ELM CONSTIT and MEL 2).

The ELM-I-1 epo-responsive erythroid progenitor cells showed fewer molecular weight variations in the representative VL30 promoter pools that were active in both untreated and epo-treated cells. Since the ELM-I-1 cell line was reported to be epo-inducible (17), the most abundant cDNA U3 promoter pool, around 300 bp, was isolated multiple times ($n$ = 16) from both the uninduced (0 and 0.5 units/mL epo) and the induced (10 units/mL epo) growth conditions. The U3 promoter clones from this pool were inserted into the TA cloning vector pCR2.1 (labeled as MEL/ELM CONSTIT; Fig. 1B, lanes 2–4). In this way, different U3 promoters within this same molecular weight pool could be delineated within the respective growth conditions. In addition, another U3 promoter cDNA pool, (350–375 bp), showed expression at inducible epo levels (10 units/mL epo). Epo-induced ELM promoters from this pool (labeled ELM 3, 4, 5, 6; Fig. 1B, lane 4) were also cloned into the TA cloning vector pCR2.1.

### *Sequence Homology Analyses of Candidate Epo-Responsive VL30 Promoters*

Six predominant VL30 promoter clones were identified (Fig. 2). Two clones were from the epo-treated MEL 585S cells (MEL/ELM CONSTIT and MEL 2; Fig. 1A, white arrow) and five clones were from the ELM-I-1 cells (MEL/ELM CONSTIT, ELM 3, ELM 4, ELM 5, and ELM 6; Fig. 1B, black arrows). The MEL/ELM CONSTIT clone was designated because the most actively expressed ELM VL30 promoter (305 bp) was 100% conserved in sequence between the uninduced ELM clones (0 and 0.5 units/mL epo, $n$ = 8) and epo-induced ELM clones (10 units/mL epo, $n$ = 8) as well as a clone isolated from the epo-treated MEL erythroid progenitor cell line ($n$ = 4). This promoter showed closest homology (91%) to a mouse cDNA clone (BE948973.1) and

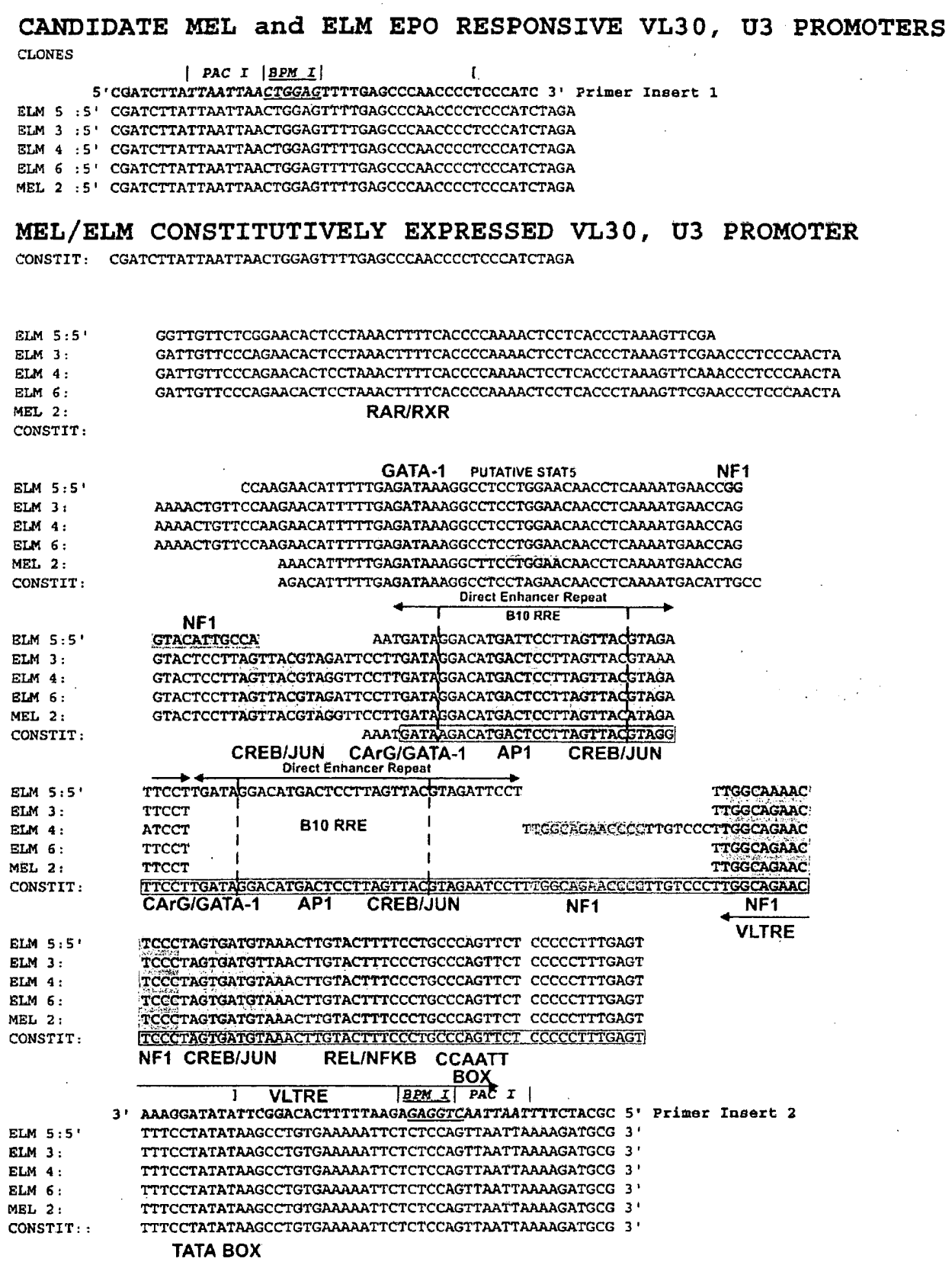


**FIG. 2.** Sequences of candidate erythropoietin (Epo)-responsive U3, VL30 promoters. RT-PCR insert primers 1 and 2 are shown on the respective 5′ and 3′ ends of the RT-PCR products. Putative transcription factor binding sites RXR/RAR, NF1, CREB/Jun, CArG element, AP1, c-Rel and p65 (REL/NFκB), are identified as well as the direct enhancer repeat regions and the VL30 12-*O*-tetradecanoylphorbol-13-acetate, (TPA) response element, (VLTRE). GATA-1 binding sites are positioned within the CArG element site. JAK2/STAT5 and B10 Ras-responsive element (B10 RRE) sites are indicated. The region boxed within the MEL/ELM CONSTIT sequence represents the ribonucleotide sequence protected by the ribonuclease protection assay probe, 151 bases.

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was grouped with members of the VL30 subgroup IV, a subgroup shown to be transcriptionally active in mouse heart and muscle RNA (13). The other five were categorized in subgroup III (13), a subgroup shown to be transcriptionally active in testis, kidney, liver, lung, spleen, and heart tissue types (13). The MEL 2 promoter sequence (270 bp) displayed a JAK2/STAT5 recognition sequence (TTCCTGGAA); however, this sequence showed 100% homology to a mouse mammary gland cDNA (BE626133.1). The ELM clones contained a putative JAK2/STAT5 recognition site with eight of the nine bases accounted for in the consensus sequence (NTCCTGGAA). ELM 3 and 6 (355 bp) both showed 98% homology to a kidney cDNA clone (AI788547.1). ELM 4 (375 bp) showed 99% homology to a mouse cDNA clone (W82836.1). Only ELM 5 (356 bp) showed homology (97%) to a previously identified VL30 element, BVL-1 (X17124) (18), with potential for epo-induced expression (14).

Putative transcription factor binding sites such as NF1, AP1, and CREB/Jun (13) were identified as well as erythroid specific GATA-1 (19) and Jak2/STAT5 consensus binding motifs (20). Both the ELM 5 and MEL/ELM CONSTIT promoters contained two B10 Ras-responsive elements (B10 RRE), (GGACATGACTCCTTAGTTAC) within their direct enhancer repeat regions (10). We focused on quantitating the respective enhanced or sustained expressions of those promoters that were isolated from the ELM-I-1 and MEL cells grown in epo treated growth conditions, specifically, (MEL 2, ELM 3, 4, 5, and 6) as well as the promoter that was shown to be a conserved VL30 clone, MEL/ELM CONSTIT.

### *Ribonuclease Protection of Epo-Inducible VL30 Elements*

Transcriptional activation of the epo inducible VL30 elements, as well as the conserved MEL/ELM CONSTIT clone were assayed through ribonuclease protection assays (RPAs), and quantified on a GS-525 molecular imager system (Bio-Rad). Figure 3 shows the data from the RPAs of the ELM-I-1 RNA. An antisense probe (Fig. 2; boxed region, Fig. 3, lane 3) was able to detect fully hybridized VL30 transcripts (MEL/ELM CONSTIT) as well as partially hybridized VL30 transcripts (MEL 2, ELM 3, ELM 4, ELM 5, and ELM 6). A control actin probe (Fig. 3, lane 8) was also hybridized to the respective total RNA samples, Band E (Fig. 3, lanes 6 and 7). Ribonuclease protection of epo induced VL30s, including ELM 3, 5, and 6, as shown through the truncated 62 bp fragment, Band A (NF1, CREB/Jun, REL/NFK$\beta$, and CCAATT Box) exhibited a 1.5-fold enhanced protection ($P = 0.0344$, $n = 5$). Identical sequence homology between the epo inducible promoters prevented discrimination between the ELM 5 promoter's expression from ELM 3 or ELM 6. The truncated 82-bp fragment, Band B corresponded to the ELM 4 VL30 and showed a 1.4-fold enhancement of hybridization ($P = 0.0406$, $n = 5$). Band C, the 116-bp truncated product, may have also been derived from partial probe hybridization to ELM 4, and showed a 1.7-fold enhancement of hybridization ($P = 0.0353$, $n = 5$). The fully hybridized antisense probe, Band D, exhibited a 1.5-fold enhanced protection of MEL/ELM CONSTIT ($P = 0.034$, $n = 5$) (Fig. 3, lanes 4 and 5). In contrast, RPAs from MEL cells showed unaltered expression (data not shown). While the MEL RNA exhibited complete hybridization of the fully hybridized antisense probe, Band D, to VL30 MEL/ELM CONSTIT-like sequences under both untreated and epo-treated growth conditions, quantitation showed that the hybridizations were not quite significantly altered in their expression ($P = 0.0637$, $n = 4$). Evidence of partial hybridization of the probe to the potential MEL VL30 transcripts such as MEL 2 was evident through the appearance of the 62-bp fragment. These transcripts showed a 1.1-fold enhanced ribonuclease protection in the epo treated conditions after 2 h ($P = 0.0109$, $n = 3$), however did not show enhanced protection, like the ELM cells following a 3-day epo treatment ($P = 0.2465$, $n = 3$).

## DISCUSSION

We sought to isolate erythroid specific VL30 promoters from erythroid progenitor cells treated

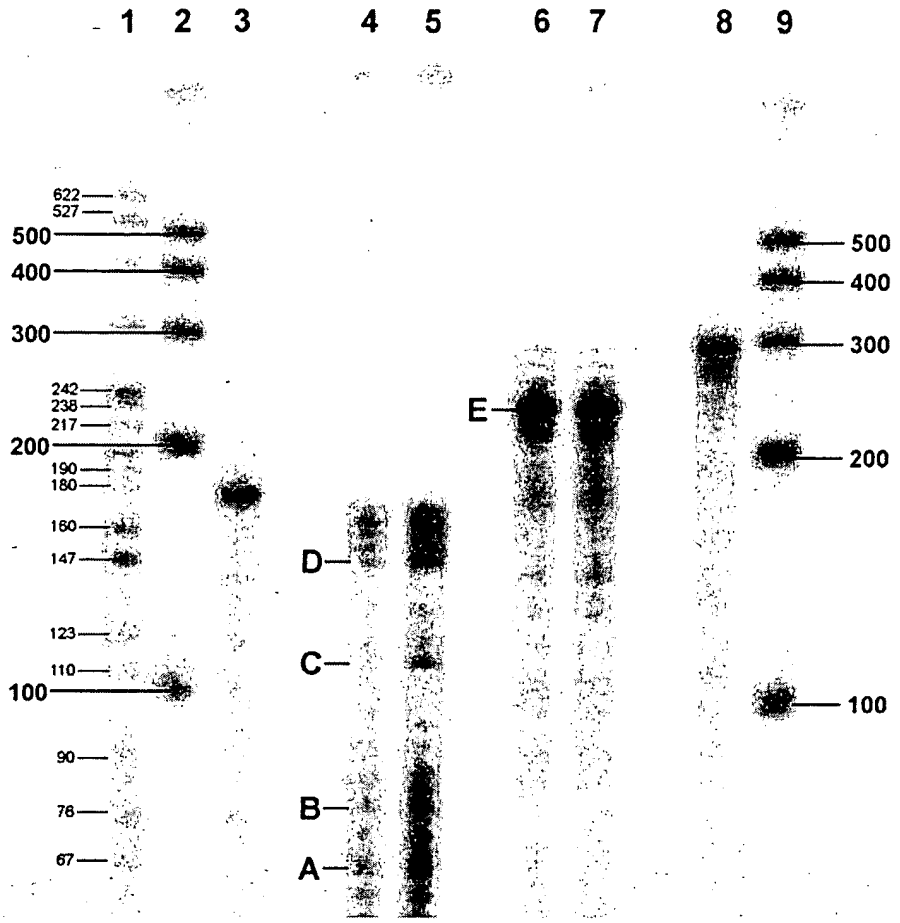


**FIG. 3.** Ribonuclease protection of Epo-treated VL30 promoters. Ribonuclease protection assays were performed on endogenous total RNA from the ELM-I-1 cell line. Nucleic acid sizes are shown by the *Msp*I-digested pBR322 ladder (lane 1) and 100-base RNA transcripts (lanes 2 and 9). Lane 3, VL30 probe alone, 177 bases. The VL30 antisense probe partially hybridized to the respective epo-induced promoters (band A, 62 bases, ELM 5/ELM 3/ELM 6; band B, 82 bp, ELM 4; band C, 116 bp, ELM 4) from uninduced (lane 4) and epo-treated (lane 5) total RNA (5 $\mu$g) is shown. The fully hybridized RPA product is indicated and corresponds to probe hybridization to MEL/ELM CONSTIT (band B, 151 bases, lanes 4 and 5). Lane 8 shows the mouse actin probe alone, 304 bases. Control lanes 6 and 7 show the probe hybridized to actin transcripts in uninduced (lane 6) and epo-induced (lane 7) total RNA (5 $\mu$g) band E, 245 bases.

with the terminally differentiating cytokine, epo (21). MEL 585S and ELM-I-1 cells, which differ in their responsiveness to epo, were screened for potential VL30 clones using RT-PCR, and were quantitated for the potential responsiveness to epo using a ribonuclease protection assay. Previous investigations implicated only BVL-1 (18) as being epo inducible (14). RPA analyses showed that an epo-inducible U3 promoter, ELM 5, showing 97% homology to BVL-1, as well as VL30 promoters similar to ELM 5, such as ELM 3, 4, and 6, were transcriptionally activated, albeit at low levels (1.5- to 1.7-fold). Thus, BVL-1 may be a conserved, epo-inducible promoter. In addition, a transcriptionally active VL30 promoter, MEL/ELM CONSTIT, was also shown to be transcriptionally active and minimally epo inducible (1.5-fold) in ELM cells but showed no inducible characteristics in the epo-unresponsive MEL cells. The consistent activations of these retrotransposable elements following treatment with a specific cytokine such as epo supports the hypothesis that VL30s are transcriptionally activated by extracellular signals (1). *Cis*-acting composite response elements within the VL30 U3 promoters allow for this transcriptional regulation (2, 9). Epo-inducible composite response elements within VL30 promoters, such as ELM 5 (BVL-1-like), may be

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suitable in combination with the current globin gene therapy systems, to provide sustained erythroid cell specific expression (22, 23).

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## REFERENCES

1. French, N. S., and Norton, J. D. (1997) Structure and functional properties of mouse VL30 retrotransposons. *Biochim. Biophys. Acta* **1352,** 33–47.
2. Wolgamot, G., Bonham, L., and Miller, D. A. (1998) Sequence analysis of *Mus dunni* endogenous virus reveals a hybrid VL30/gibbon ape leukemia virus-like structure and a distinct envelope. *J. Virol.* **72,** 7459–7466.
3. Norton, J. D., and Hogan, B. L. (1988) Temporal and tissue-specific expression of distinct retrovirus-like (VL30) elements during mouse development. *Dev. Biol.* **125,** 226–228.
4. Hodgson, C. P., Zink, M., and Xu, G. (1996) Structure and function of mouse VL30 sequences. *In* Retro-Vectors for Human Gene Therapy (Hodgson, C. P., Ed.), pp. 73–102. Chapman & Hall, New York.
5. van der Houven van Oordt, C. W., Schouten, T. G., van der Eb, A. J., and Breuer, M. L. (1999) Differentially expressed transcripts in X-ray-induced lymphomas identified by dioxygenin-labeled differential display. *Mol. Carcinog.* **24,** 29–35.
6. Eaton, L., and Norton, J. D. (1990) Independent regulation of mouse VL30 retrotransposon expression in response to serum and oncogenic cell transformation. *Nucleic Acids Res.* **18,** 2069–2077.
7. Singh, K., Saragosti, S., and Botchan, M. (1985) Isolation of cellular genes differentially expressed in mouse NIH3T3 cells and a simian virus 40-transformed derivative: Growth-specific expression of VL30 genes. *Mol. Cell. Biol.* **5,** 2590–2598.
8. Wolgamot, G., and Miller, A. D. (1999) Replication of *Mus dunni* endogenous retrovirus depends on promoter activation followed by enhancer multimerization. *J. Virol.* **73,** 9803–9309.
9. Foster, D. N., Schmidt, L. J., Hodgson, C. P., Moses, L. H., and Setz, M. J. (1982) Polyadenylated RNA complementary to a mouse retro-virus like multigene family is rapidly and specifically induced by epidermal growth factor stimulation of quiescent cells. *Proc. Natl. Acad. Sci. USA* **79,** 7317–7321.
10. Nilsson, M., Ford, J., Bohm, S., and Toftgard, R. (1997) Characterization of a nuclear factor that binds juxtaposed with ATF3/Jun on a composite response element specifically mediating induced transcription in response to an epidermal growth factor/Ras/Raf signaling pathway. *Cell Growth Differ.* **8,** 913–920.
11. Harrigan, M. T., Baughman, G., Campbell, N. F., and Bourgeois, S. (1989) Isolation and characterization of glucocorticoid and cAMP-induced genes in T lymphocytes. *Mol. Cell. Biol.* **9,** 3438–3446.
12. Islam, T. C., Bugge, T. H., and Bohm, S. (1993) The long terminal repeat of VL30 retrotransposons contains sequences that determine retinoic acid induced transcription in cultured keratinocytes. *J. Biol. Chem.* **268,** 3215–3259.
13. Nilsson, M., and Bohm, S. (1994) Inducible and cell type-specific expression of VL30 U3 subgroups correlate with their enhancer design. *J. Virol.* **68,** 276–288.
14. Park, D. J., Lim, R. W., and Kim, H. D. (1993) Rapid induction of mouse virus-like (VL30) element transcripts by erythropoietin in murine erythroid progenitor cells. *Blood* **82,** 77–83.
15. Baron, M. H., and Maniatis, T. (1986) Rapid reprogramming of globin gene expression in transient heterokaryons. *Cell* **46,** 591–602.
16. D'Andrea, A. D., Lodish, H. F., and Wong, G. G. (1989) Expression cloning of the murine erythropoietin receptor. *Cell* **57,** 277–285.
17. Iwatuski, K., Endo, T., Misawa, H., Yokouchi, M., Matsumoto, A., Ohtsubo, M., Mori, K. J., and Yoshimura, A. (1997) STAT5 activation correlates with erythropoietin receptor-mediated erythroid differentiation of an erythroleukemia cell line. *J. Biol. Chem.* **272,** 8149–8152.
18. Hodgson, C. P., Fisk, R. Z., Arora, P., and Chotani, M. (1990) Nucleotide sequence of mouse virus-like (VL30) retrotransposon BVL-1. *Nucleic Acids Res.* **18,** 673.

19. Orkin, S. H. (1992) GATA-binding transcription factors in hematopoietic cells. *Blood* **80,** 575–581.
20. Imada, K., and Leonard, W. J. (2000) The Jak-STAT pathway. *Mol. Immunol.* **37,** 1–11.
21. Klingmuller, U. (1997) The role of tyrosine phosphorylation in proliferation and maturation of erythroid progenitor cells-signals emanating from the erythropoietin receptor. *Eur. J. Biochem.* **249,** 637–647.
22. Solaimain, F., Zink, M. A., Xu, G., Grunkemeyer, J., Cosgrove, D., Saenz, J., and Hodgson, C. P. (2000) Molecular retro-vectors for transgenic and therapeutic use. *Mol. Reprod. Dev.* **56,** 309–315.
23. Lopez-Lastra, M., Ulrici, S., Gabus, C., and Darlix, J. L. (1999) Identification of an internal ribosome entry segment in the 5′ region of the mouse VL30 retrotransposon and its use in the development of retroviral vectors. *J. Virol.* **73,** 8393–8402.

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# Sustained tissue -specific transgene expression from a vl30 retrotransposon-derived vector *in vivo*

**Research Article**

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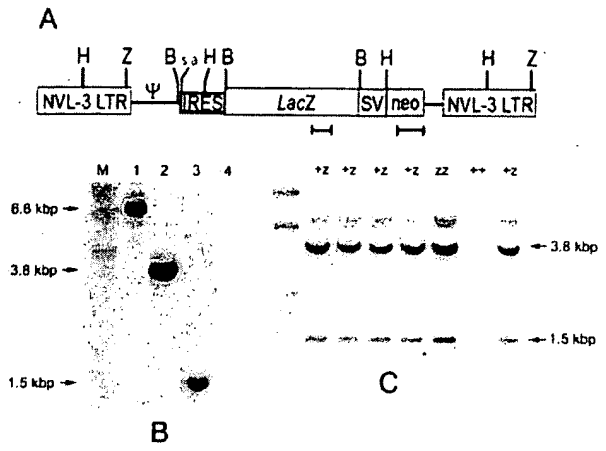
## Summary:

**Previous attempts to generate transgenic mice via retroviral transduction of pre-implantation embryos have usually not resulted in stable transgene expression. In these cases, inactivation of the retroviral LTR is associated with passage through the germ line. A subset of endogenous murine retrovirus-like retrotransposons (VL30's) are constitutively expressed in virtually all tissues, with no deleterious effects to the health of the animal. We surmised that these VL30s might be useful as vectors for stable lineage-specific transgene expression. A mouse VL30-derived retro-vector engineered to express a reporter gene (*LacZ*) was used to generate transgenic mice via transduction of embryonic stem (ES) cells. A single copy of the vector was stably integrated into a unique site in the mouse genome. Sustained tissue specific expression was observed at both the mRNA and protein levels for several generations. Transgene expression was observed in distinct sub-populations of cells in both lung and spleen. In the lung, cells expressing the vector were identified as type II pneumocytes. These data illustrate for the first time that a VL30 LTR (NVL-3) is unique from its retroviral counterparts in that it can pass through the germ line repeatedly without undergoing transcriptional inactivation. Thus, VL30 vectors may be useful for both transgenesis and as alternatives to existing retroviral vectors for gene therapy.**

## I. Introduction

Currently, methods for deriving transgenic animals rely predominantly on the direct injection of DNA into the pronuclei of oocytes (Palmiter *et al.*, 1982; Lacy *et al.* 1983). This method results in the integration of random concatamers of the original construct with varying copy number. This characteristic frequently confounds the interpretation of the results, requiring the analysis of a large number of founders to ensure correct interpretation of the data (Si-Hoe *et al.*, 1999).

An alternative approach was envisioned over two decades ago, involving the integration of a single copy retroviral vector into the germ line (Jaenisch, 1976). These studies, which employed Moloney murine leukemia virus (MoMLV), illustrated that these viruses failed to be expressed in the resulting animals. Infection of post-implantation embryos, however, does provide long-term expression of the transgene (Jahner *et al.*, 1982). Failure of transgene expression in pre-implantation embryos is linked to lack of LTR enhancer activity (Brinster *et al.*, 1981; Linney *et al.*, 1984), binding of transcriptional repressors to the retrovirus tRNA primer binding site (Loh *et al.*, 1987, 1988; Peterson *et al.*, 1991), and possibly to DNA methylation of retroviral sequences as well as DNA flanking the integration site (Barker *et al.*, 1991; Hoeben *et al.*, 1991; Jahner *et al.*, 1985).




**Figúre 1.** Production of a transgenic mouse line harboring the VLSAIBAG retrotransposon construct. The synthetic retrotransposon, VLSAIBAG, engineered for these studies is illustrated in panel A. Positions of the NVL-3 long terminal repeats (NVL-3 LTR), *Psi* packaging sequences (Ψ), splice acceptor site (s.a.), internal ribosome entry site (IRES), β-galactosidase expression cassette (*Lac-Z*), SV40 virus early region promoter (SV) and neomycin phosphotransferase expression cassette (*neo*) are shown. The positional locations of probes used are indicated by bars. Key restriction endonuclease recognition sites are indicated by letters: H= *Hind*III; Z= *Xho*I; B=*BamH*I. Panel B is a Southern blot illustrating single copy integration of an intact vector in the ES cell clone used to generate transgenic animals. Lanes: M, marker; l-*Hind*III; 1, *Xho*I digest hybridized with *lac*Z probe; 2, *Hind*III digest hybridized with *lac*Z probe; 3, *Hind*III digest hybridized with *neo* probe; 4, untransduced ES cell DNA; 5, EcoRV digest hybridized with *Lac-Z* probe. Panel C illustrates typical results for genotype analysis by Southern blot of a litter of pups derived by crossing two mice heterozygous for the transgene. Genotypes are listed above each lane. ++ = normal mouse; +Z = hemizygous for the transgene; ZZ = homozygous for the transgene.

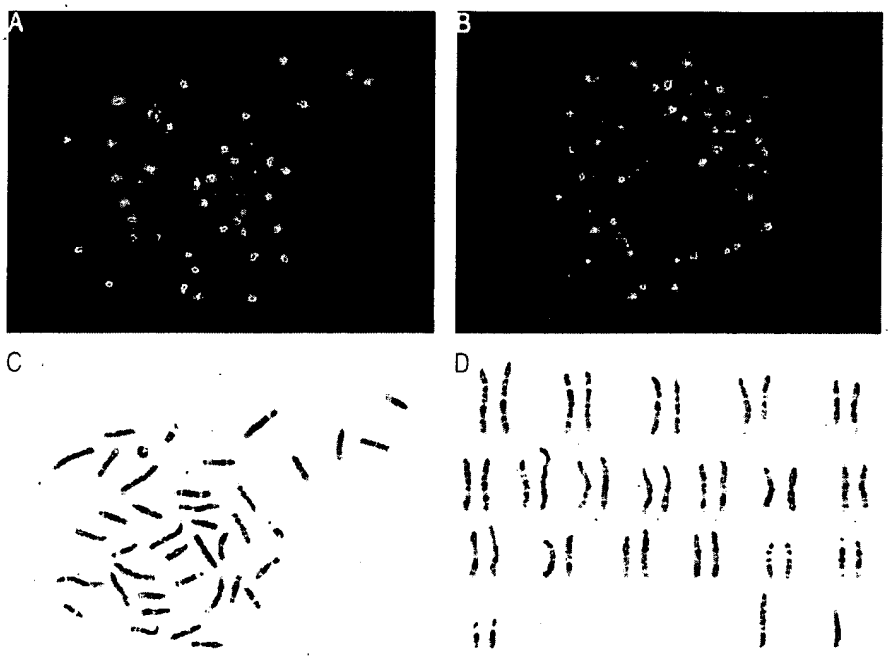



**Figure 2.** Cytogenetic analysis of VLSAIBAG integration site. (A and B) FISH signal showing vector integration site in two different metaphase spreads from splenocytes of homozygous transgenic mice. (C) G-banding analysis of same spread as in A. Chromosomes with fluorescent signal are indicated by arrows. (D) Typical mouse idiogram as reference for panel C.

Related to the murine type C retroviruses is a family of long terminal repeat- (LTR) bearing retrotrotransposable elements (VL30's) present at 100 to 200 copies in the genomes of most species (Keshet *et al.*, 1980; Courtney *et al.*, 1982) [14,15]. Subsets of these endogenous elements are naturally expressed in many tissues (Norton *et al.*, 1988; Nilsson and Bohm, 1994). During retroviral infections, mouse VL30 RNA is efficiently transmitted by pseudotyping (co-packaging) into retrovirus particles (Howk *et al.*, 1978; Besmer *et al.*, 1979; Scolnick *et al.*, 1979). Vectors derived from murine VL30's are expressed in a variety of mammalian cell types, both primary and transformed (Chakraborty *et al.*, 1993 & 1995).

These characteristics of VL30 suggested that vectors derived from these elements might give sustained expression *in vivo*. To test this hypothesis, a construct expressing *LacZ* from a VL30 LTR was packaged using a standard retroviral packaging cell line, and the resulting virions were used to transduce murine embryonic stem cells. Selected transductants were used to derive a transgenic animal via injection of ES cells into 3.5 day blastocysts and surgical reimplantation. Sustained and tissue specific expression was observed in the resulting transgenic mouse line. These results illustrate the utility of VL30 derived retro-vectors for obtaining transgene expression *in vivo*.

## II. Results

### A. Derivation of vector and construction of the transgenic mouse line

The vector used in these studies, which is referred to as VLSAIBAG (**Fig. 1A**), was derived from the endogenous VL30 element NVL-3 (Carter *et al.*, 1983). It contains a *LacZ* reporter gene expressed from the NVL-3 long terminal repeat (LTR). As VL30 RNA is poorly translated into protein (unpublished observations), an internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV) was cloned 5' of the *LacZ* open reading frame to ensure efficient translation of the reporter construct (Grunkemeyer *et al.*, submitted). Embryonic stem cells were transduced using viral supernatants from PA317/VLSAIBAG vector producer cells (VPCs). A number of clonal cell lines were obtained by G418 drug selection. One such clone was selected for micro-injection into mouse blastocysts, resulting in the production of a single transgenic mouse line. The transgene was integrated as a single copy, without rearrangements, in both the ES cells (**Fig 1B**) and in the resulting transgenic animals (**Fig. 1C**). Fluorescence *in situ* hybridization (FISH) analysis demonstrated that a single autosomal integrant was stable through several mouse generations (**Fig. 2**).

### B. Analysis of vector expression *in vivo*

Preliminary screening was carried out by reverse transcriptase polymerase chain reaction (RT-PCR) on total RNA from various embryonic and adult tissues. These analyses indicated expression in both spleen and lung (data not shown). RNase protection analysis was then performed using total RNA isolated from lung and spleen tissue from four and 15 week old animals. **Fig. 3A** illustrates that a 194 bp protected fragment (corresponding to a portion of the *lacZ* structural gene) was present in both lung and spleen tissue. Significant amounts of vector RNA were observed in samples derived from both young (29 day old) and mature (99 day old) mice, illustrating that expression of the reporter gene is stable. To verify that the probe was detecting full-length transcripts of the appropriate molecular size, RNA blot analysis was performed using total RNA from lung tissue. **Fig. 3B** demonstrates that the appropriate sized transcript (6.6 kb) was expressed in lung. Vector-derived transcript levels in the spleen were too low to be detected by this method.

Expression of vector-derived transcripts in RNA preparations from whole tissues might be derived from a specific cellular compartment within the tissue. To delineate transgene expression at the cellular level, we employed a histochemical staining technique for enzymatically active β-gal protein. Results shown in **Fig. 4** demonstrated significant transgene expression in a subset of cells in both lung (**Fig. 4B**, and **D**) and spleen (**Fig. 4F**). Neither the number of cells nor the intensity of staining appeared to vary significantly between lung tissue derived from animals of two-weeks (**Fig. 4B**) versus 10-weeks (**Fig. 4D**) of age. No staining was observed in these same tissues from age-matched non-transgenic littermates (**Fig. 4A, C** and **E**).

Based upon the morphology, frequency, and localization of β-gal positive staining cells of the lung, we surmised that they may be either macrophages or type II pneumocytes. To resolve this issue, tissues were first stained with X-gal, followed by staining of sections with antibodies specific for either macrophages or type II pneumocytes. The same fields were observed by both light and fluorescence microscopy to determine the identity of β-gal positive cells. Analysis of **Fig. 5A**, and **5B** clearly illustrates that cells staining positive for the macrophage marker are not positive for β-galactosidase activity. Similar analysis was carried out using an antibody specific for the pro-peptide form of human surfactant protein C, which is an established marker for type II pneumocytes (Vorbroker *et al.*, 1995). Comparison of **Fig. 5C** with **Fig. 5D** illustrates that all blue cells were also positive for surfactant protein C, identifying these β-gal positive cells as type II pneumocytes. It should be noted, however, that not all antibody positive cells were blue, suggesting the transgene was expressed in a sub-population of type II pneumocytes in the lung.

Similar attempts to identify the *Lac-Z* positive cells in

the spleen were inconclusive.

## III. Discussion

The results described herein demonstrate that transgenic animals can be derived via transduction of mouse embryonic stem cells using retro-vectors engineered to express heterologous gene products from at least one VL30 promoter (NVL-3). In the example described, a stably transduced ES cell clone was used to generate a transgenic mouse which expressed theVL30 vector, VLSAIBAG, in a tissue-specific manner. The retro-vector was integrated in single copy, and shown to pass through the germ-line several times (>5) without affecting either the expression levels or the cellular specificity of expression. Earlier attempts to derive

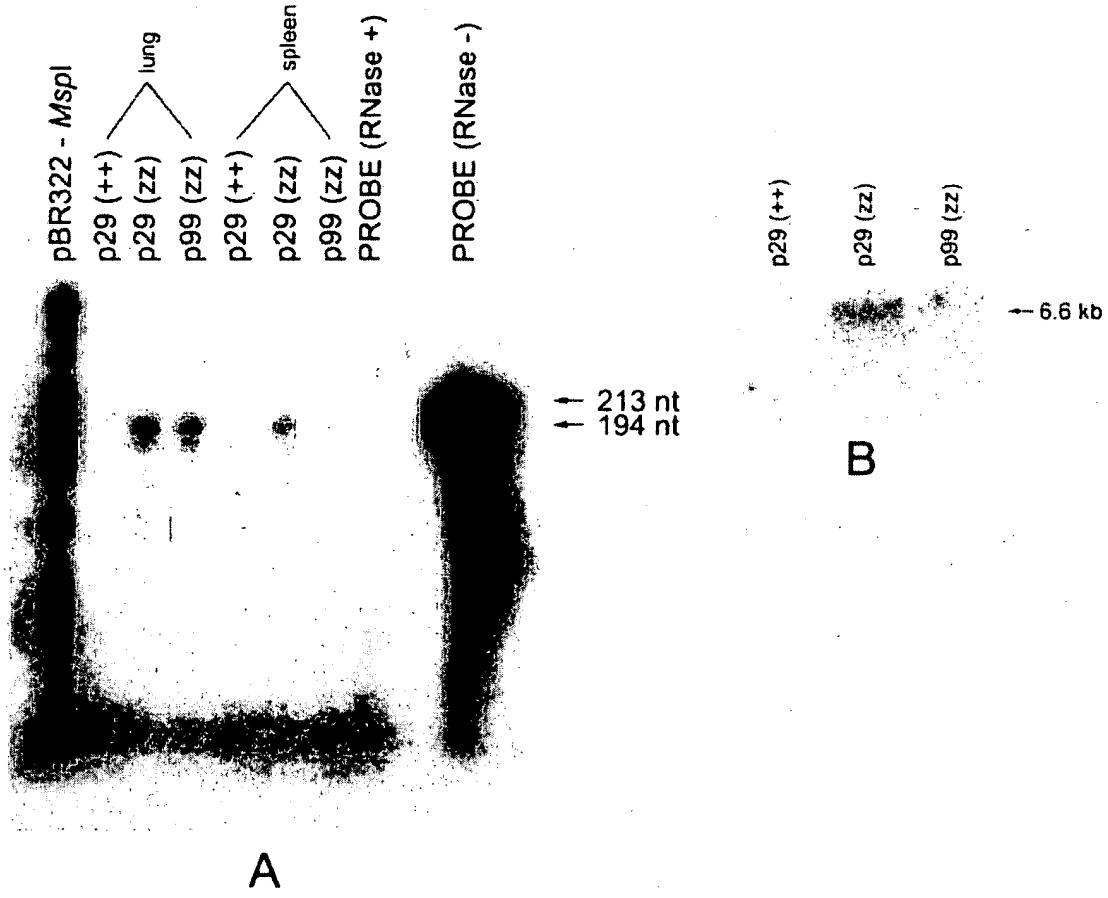



**Figure 3.** Expression of LTR-driven transcripts from lung and spleen in transgenic mice. Panel A is RNase protection analysis of total cellular RNA from both lung and spleen. Total RNA from either 29 day-old (p29) or 99 day-old (p99) mice were analyzed using a 213 base pair riboprobe. The expected size of the protected fragment was 194 base pairs. Probe (RNase +) is probe digested with ribonuclease; probe (RNase -) is undigested probe. (++) are wild type controls; (ZZ) are mice homozygous for the transgene. Panel B represents a northern blot of total lung RNA from either 29 day-old (p29) or 99 day-old (p99) transgenic mice hybridized with a probe derived from the *LacZ* structural gene (**Fig. 1A**). The expected size band is visible at 6.6 kb.

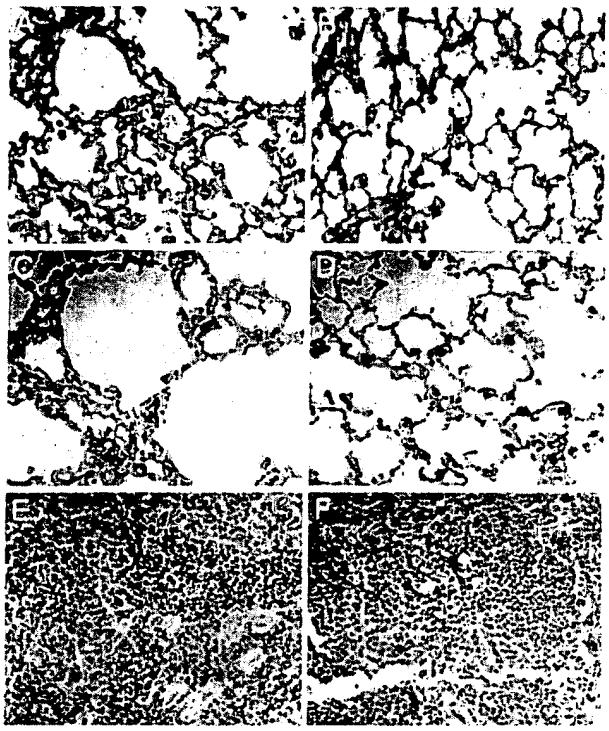


**Figure 4.** Expression of the VL30 transgene at the cellular level in lung and spleen. Tissues were stained histochemically for β-galactosidase expression, then embedded in historesin plastic, and cut at 2.5 μM. Sections were counter-stained with eosin and hematoxylin and photographed at 200 X magnification. Panels A and B show lung from 15 day old normal (A) and homozygous transgenic (B) mouse. Panels C and D show lung from 71 day old non-transgenic (C) and homozygous transgenic (D) mice. Panels E and F show spleen from 71 day old normal (E) and homozygous transgenic (F) mice.

transgenic mice from embryonic cells transduced with retroviral vectors resulted in inactivation of expression after passage through the germ line (Jahner and Jaenisch, 1985). Inactivation was attributed to methylation of both the retroviral genome and the DNA surrounding the site of integration, based on direct studies as well as experiments where the retroviral genome was activated by chemical demethylation of the DNA with 5-azacytidine (Jahner *et al.*, 1982; Jahner and Jaenisch, 1985). Thus, this method for producing transgenic animals has been largely abandoned. VL30-derived vectors offer the opportunity to revisit this strategy.

The use of retroviral transduction of embryonic stem cells as a means of generating transgenic animals has obvious advantages over methods currently employed. The vector can be inserted in a single copy, avoiding complications due to concatamerization of the transgene. It allows for testing of gene therapy vectors *in vivo*, allowing quick assessment of tissue specificity and toxicity. There are more than 100 copies of VL30 in the mouse genome with wide ranging developmental and tissue specific expression patterns (Sanes *et al.*, 1986; Norton and Hogan, 1988; Nilsson and Bohm, 1994), and inducibility (Rodland *et al.*, 1986, 1988; Lenormand *et al.*, 1992), suggesting that their use in transgenics might have broad applicability.

In an earlier report, Nilsson and Bohm, 1994, examined the endogenous expression patterns for a number of the known VL30 elements in mouse tissues. They demonstrated expression of the subclass of VL30 LTR's that includes NVL-3 are expressed in the lung and spleen. Our construct employed the NVL-3 LTR, and as reported herein, was expressed in both the lung and the spleen. Nilsson and Bohm did not resolve expression at the cellular level in these tissues. Our results suggest that the NVL-3 LTR is regulated in the appropriate developmental and tissue specific manner in the transgenic mouse line. However, because only one transgenic line was analyzed, it is not possible to say with certainty that the transgene functioned in a position-independent manner. Generation and analysis of more transgenic lines, derived from indepentdently-transduced ES cell clones, are necessary to resolve this issue. Indeed, expression of other retro-vectors have been shown to be sensitive to position effects (Hoeben *et al.*, 1991). However, the fact that expression was observed in the same two tissues as observed previously by Nilsson and Bohm strongly suggests that the LTRs are specific for these tissues.

This is the first time that tissue-specific expression of a transgene has been demonstrated using a VL30-derived vector. These findings point toward the use of VL30-derived vectors for the generation of transgenic animals expressing a heterologous gene in a tissue-specific or developmental stage-specific pattern. The large number of endogenous VL30 LTRs which have been shown to be expressed in distinct tissues at various times of development, may serve as a reservoir of promoters for transgenic constructs as well as potentially being useful for gene therapy.

Expression of the retro-vector in type II pneumocytes illustrates highly specific cellular expression of the NVL-3 LTR promoter. Indeed, only a subset of pro-SPC-positive type II pneumocytes were positive for _-gal staining. Currently, pro-SPC is an accepted marker for the identification of type II pneumocytes (Vorbroker *et al.*, 1995). Our data suggests that the capacity to express the retro-vector may delineate a subtype of type II pneumocyte or simply that vector expression in some type II pneumocytes is too low to be detected by the employed methods. Targeting expression to type II pneumocytes might provide therapeutic angles for diseases such as pulmonary emphysema, forms of which have been attributed to defects in matrix metalloproteinase expression (Ohnishi *et al.*, 1998).

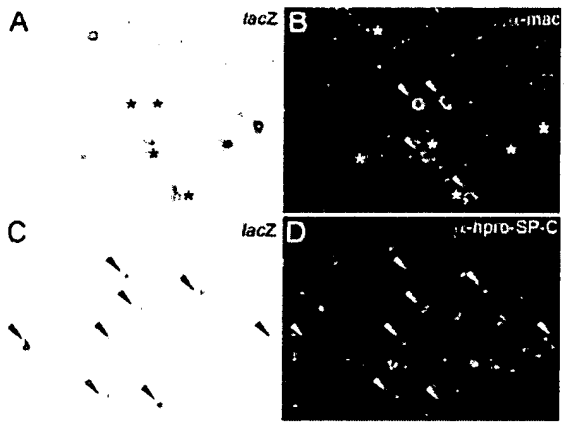



**Figure 5.** Identification of β-galactosidase positive cells in the lung as type II pneumocytes. Lung tissue from a 71 day old homozygote was stained for β-gal expression, embedded in aqueous mounting medium, and cryosections stained with either α-Mac (a cell surface marker for macrophages, panel B), or anti-human surfactant protein C pro-peptide (α-hpro-SP-C, a marker for type II pneumocytes, panel D). The same field was recorded by light microscopy (panels A and C) for β-gal staining, and fluorescence microscopy (panels B and D) for the specific cell markers to allow identificaiton of dual positive cells as type II pneumocytes (indicated by arrows in panels C and D), and not macrophages (lack of dual positive cells indicated by non-overlapping asterisks and arrows in panel B).

## Acknowlegements

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# IV. Materials and Methods

## A. Vector construction

All plasmids were constructed according to standard protocols (Ausubel *et al.*, 1995). Plasmid pVLBAG was generated by cloning a blunted *lac*Z/simian virus-40 (SV40) early region transcriptional promoter/neomycin phosphotransferase resistance cassette from pDOL (Price *et al.*, 1987) into the unique *Not*I site of plasmid pVLPP (Chakrablrty *et al.*, 1993). Plasmid pVLPP is a synthetic VL30 vector containing the LTRs and *psi* packaging signal from the murine retrotransposon, NVL-3 Carter *et al.*, 1983). Plasmid pVLIBAG was then constructed using pVLBAG as vector. A blunted 601 bp *Nco*I-*Sal*I fragment (from pG1IL2EN) (Treisman *et al.*, 1995), containing the IRES sequence from encephalomyocarditis virus (Jang *et al.*, 1988), was ligated into the unique *Pac*I site in pVLBAG immediately 5' of the β-gal initiator AUG codon.This clone was used as vector for cloning pVLSAIBAG, the vector used in these studies. A 26 bp splice acceptor fragment was cloned immediately 5' of the IRES. The splice acceptor was found to be non-functional both *in vitro* and *in vivo* (unpublished observation). For RNase protection assays, plasmid pRIBOGAL was constructed by cloning 194 bp of the *lac*Z coding sequence into pBluescriptII (Stratagene, La Jolla, CA). All clones were verified by restriction and sequence analysis.

## B. Viral transductions

The embryonic cell line RW4 (Genome Systems, St. Louis, MO) was cultured according to the method of Robertson, 1987. ES cells were transduced by supernatants according to standard protocols (Cepko et al, 1995). Viral supernatants were harvested from rapidly growing (just confluent) cultures of PA317s, filtered through 0.2 μ filter (Nalgene, Milwaukee, WI), and added to ES cell cultures which had been passed onto gelatinized plates. The following day, the cells were washed twice with PBS and allowed to grow for 48 hours. Transduced ES cells were selected in 175 μg/ml G418 for 10 days. G418 resistant clones were expanded, and DNA (Puregene kit; Gentra Systems, Inc.,

Research Triangle Park, NC) was isolated for determination of vector integrity (no major rearrangements or deletions) and copy number. Clonal cell lines with correct morphology and a single integrated copy of VLSAIBAG were expanded for injection into pre-implantation embryos.

## C. Genetic typing

Hemizygote (designated +Z) males and females were maintained as breeders in the colony so that each litter had a likelihood of providing wild-type (++) and homozygous (ZZ) animals for experimentation. DNA was isolated from tail DNA according to the following method: an approximately 1 cm piece of tail was clipped with a sterile scalpel and incubated in 0.5 ml digestion buffer (50mM Tris-Cl, pH 7.5; 50 mM EDTA; 1% SDS and 10 μg/ml Proteinase K, Boehringer Mannheim) overnight at 37°C and purified as described previously (Hogan *et al.*, 1994). Tail DNA was digested with *Hind*III, electrophoresed, and hybridized to $^{32}$P-labelled probes for both the *lacZ* and *neo* coding sequences. After exposure of autoradiographic film, blots were exposed to a phospor screen to quantify the amount of signal in each lane and enable the distinction of hemi- and homozygous animals.

## D. Fluorescence *in situ* hybridization analysis

Cells were prepared for FISH and G-banding (see legend for **Fig. 2C**) analysis as previously described (Takashi *et al.*, 1991). Briefly, splenocytes were cultured for 2-4 days in the presence of concanavalin A and lipopolysaccharide to stimulate cell division. Metaphase spreads were prepared by synchronizing the cells with 300 _g/ml thymidine, culturing in 5-bromo-deoxyuridine (BrdU) and arresting them at metaphase with colchicine. The spreads were subjected to FISH analysis as described.

The spreads were heat denatured and hybridized to a digoxigenin-labelled probe (dig-dUTP; Boehringer Mannheim), which was generated by nick translation of the *lacZ* probe (used for Southern and Northern analyses). After hybridization, slides were washed and incubated with a fluorescein-conjugated anti-digoxigenin antibody. The slides were viewed on an Olympus BH-2 microscope equipped for epi-illumination. DAPI (4,6 Diamidino-2-phenylindole) was used as a counterstain for the chromosomes (excitation = 367 nm, emission = 453 nm). FITC (Fluoroscein isothiocyanate) was used to label the probe (excitation = 497 nm, emission = 524 nm). Low light level fluorochrome signals were captured and enhanced using the Cytovision system (Applied Imaging Inc., Pittsburgh, PA).

## E. Northern blot and RNase protection analyses

Isolation of RNA from tissues was done using TRIzol™ reagent (InVitrogen Corp, Gaithesburg, MD) according to the manufacturers instructions. Total RNA was fractionated by electrophoresis on agarose-formaldehyde gels (20 μg sample/lane). Blots were UV-crosslinked, air-dried and hybridized to a probe for the *lacZ* coding sequence. RNase protection assays were performed using the same total cellular RNA used for Northern analyses with a kit (RPA II kit) from Ambion, Inc. (Austin, TX) according to the manufacturer's instructions. Antisense riboprobes were synthesized *in vitro* using T3 RNA polymerase (Boehringer Mannheim), labeled $\alpha^{32}$P-UTP (Amersham), and *linearized* plasmid pRIBOGAL. The riboprobe contains extra vector sequences, so undigested probe (213 bases) can be differentiated from fully protected probe (194 bases) on a denaturing acrylamide gel.

## F. Histochemical stain for β-gal activity and visualization

Histochemical staining of tissues with X-gal was performed as previously described (Sanes *et al.*, 1986). Tissues were fixed in 4% paraformaldehyde (PFA, Sigma Chemical Co.) for 1-2 hours at room temperature. After fixation, tissues were rinsed four times in PBS and incubated overnight at 30°C in X-gal stain solution [5.0 mM $K_3Fe(CN)_6$; 5.0 mM $K_4Fe(CN)_6$; 1.5 mM $MgCl_2$; 0.02% NP-40; 0.01% sodium deoxycholate with 1.0 mg/ml X-gal (in DMSO) in PBS]. After staining, tissues were rinsed in PBS and embedded in Historesin using the methods described by the manufacturer (Leica; Heidelberg, Germany). Tissue blocks were sectioned at 2.5 μm with a Sorvall JB-4 microtome (Ivan Sorvall, Inc., Newton, CT) using a glass knife. To visualize cell morphology of cells stained blue (due to β-gal activity) in the context of the surrounding cells, each section was stained with Harris' hematoxylin and counterstained in alcoholic eosin according to standard protocols (Allen *et al.*, 1992). Sections were coverslipped with an organic mounting medium (Curtin Matheson Scientific, Inc., Houston, TX) and visualized on an Olympus BH2 oil immersion microscope fitted with a digital camera and imaging system (Cytovision, Applied Imaging, Pittsburgh, PA).

## G. Immunohistochemical staining and visualization

Tissues stained with X-gal were also prepared for immunohistochemical analysis. After staining, tissues were rinsed in PBS and embedded in Tissue-Tek™ (Miles Inc., Elkhart, IN) embedding medium. Tissues were frozen and stored at -150°C and warmed to -25°C prior to sectioning. Blocks were sectioned at 3 μ on a Microm HM 505 N

cryostat (Carl Zeiss, Inc., Thornwood, NY)

Various antibodies/antisera were used to identify the specific cell types that express the vector VLSAIBAG *in vivo*. A polyclonal antisera against mouse macrophages (α-mac; Accurate Chemical and Scientific Corp., Westbury, NY), and a polyclonal antibody against the pro-peptide of human surfactant protein C (α-hpro-SP-C, Vorbroker *et al.*, 1995) were used for immunohistochemical analysis on X-gal stained tissue sections. The basic method is as described (Watkins, 1989).

For the α-mac antisera, normal goat immunoglobulin G (Rabbit IgG, Vector Laboratories, Inc., Burlingame, CA) was diluted to 12.5 μg/ml in 7% non-fat dry milk in PBS (milk/PBS) and incubated on the section for 10-30 minutes at room temp. Next, the primary antibody (polyclonal antibody/antisera) was diluted (1:300 dilution) in milk/PBS and placed on each section. Sections were incubated with the primary antibody overnight at 4°C. After incubation with the primary antibody, the sections were washed three times in PBS before the FITC-conjugated (fluorescein isothiocyanate) secondary antibody was applied to the sections. The secondary antibody was diluted 1:100 in milk/PBS and incubated on each section for 2.5-3.5 hours at room temperature in the dark. Finally, the slides were washed three times in PBS and coverslipped with Vectashield™ (Vector Laboratories, Inc., Burlingame, CA) mounting medium. The slides were documented with an Olympus BH2 oil immersion microscope fitted with a digital camera and imaging system (Cytovision, Applied Imaging, Pittsburgh, PA).

For the α-hpro-SP-C polyclonal, sections were blocked (2% normal goat serum; 0.2% Triton X-100 in PBS) for 90 minutes at room temperature before the addition of α-hpro-SP-C (dilution of 1:1000 in 2% normal goat serum; 0.2% Triton X-100 in PBS). Primary antibody was incubated overnight at 4°C. The next morning, the slides were washed with 0.2% Triton X-100 in PBS before addition of the FITC-conjugated secondary diluted 1:100 in PBS containing 0.2% Triton X-100. After 2.5 hours at room temperature in the dark, slides were washed with 0.2% Triton X-100 in PBS (1 wash for 5 minutes), PBS alone (2 washes for 5 minutes each) mounted with Vectashield, coverslipped and imaged.

## References


Allen, TC (**1992**) *In* **Laboratory methods in histotechnology** (E. Prophet, B. Mills, J. G. Arrington and L. H. Sobin, eds.), pp. 56-57, American Registry of Pathology, Washington, D.C.

Ausubel, FM, Brent, R, Kingston, RE, Moore, DD, Seidman, JG, Smith, JA, and Struhl, K, eds. **1995. Current protocols in molecular biology**. Wiley and Sons, New York.

Barker, DD, Wu, H, Hartung, S, Breindl, M, and Jaenisch, R (**1991**) Retrovirus-induced insertional mutagenesis: mechanism of collagen mutation in mov13 mice. ***Mol. Cell. Biol.*** 11, 5154-5163.

Besmer, P, Olshevsky, U, Baltimore, D, Dolberg, D, and Fan, H (**1979**) Virus-like 30S RNA in mouse cells. **J. Virol.** 29, 1168-1176.

Bohm, S (**1991**) Identification of protein-binding sequences mediating constitutive and 12-o-tetradecanoylphorbol-13-acetate-induced VL30 transcription in cultured mouse and human keratinocytes. **J. Biol. Chem.** 266, 24834-24841.

Brinster, RL, Chen, HY, Trumbauer, M, Senear, AW, Warren, R, and Palmiter, RD (**1981**) Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. **Cell** 1, 223-31.

Brown, T, and Mackey, K (**1997**) Analysis of RNA by Northern and slot blot hybridization. *In* **Current Protocols in Molecular biology** (F. M. Ausubel, R. Brent, R. Kingston, D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds.), pp. 4.9.1-4.9.16, Wiley and Sons, Inc., New York, NY.

Carter AT, Norton JD, Avery RJ (1983) A novel approach to cloning transcriptionally active retrovirus-like genetic elements from mouse cells. Nucleic Acids Res. 11, 6243-6454.

Cepko, C (**1995**) Preparation of a specific retrovirus producer cell line. *In* **Current Protocols in Molecular Biology** (F. M. Ausubel, R. Brent, R. Kingston, D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds.), 1995. pp. 9.10.1-9.10.13, Wiley and Sons, Inc., New York, NY.

Chakraborty, AK, Zink, MA, and Hodgson, CP (**1995**) Expression of VL30 vectors in human cells that are targets for gene therapy. **Biochem. Biophys. Res. Commun.** 209, 677-683.

Chakraborty, AK, Zink, MA, Boman, BM, and Hodgson, CP (**1993**) Synthetic retrotransposon vectors for gene therapy. **FASEB J.** 7, 971-977.

Courtney, MG, Schmidt, LJ, and Getz, MJ (**1982**) Organization and expression of endogenous virus-like (VL30) DNA sequences in nontransformed and chemically transformed mouse embryo cells in culture. **Cancer Res.** 42, 569-576.

Hoeben, RC, Migchielsen, AAJ, van der Jagt, RCM., van Ormondt, H, and van der Eb, AJ (**1991**) Inactivation of the moloney murine leukemia virus long terminal repeat in murine fibroblast cell. **J. Virol.** 65, 904-912.

Hogan, B, Beddington, R, Costantini, F, and Lacy, E (**1994**) Manipulating the mouse embryo, a laboratory manual, Second ed., Cold Spring Harbor Press, Plainview, NY.

Howk, RS, Troxler, DH, Lowy, D, Duesberg, PH, and Scolnick, EM (**1978**). Identification of a 30S RNA with properties of a defective type c virus in murine cells. **J. Virol.** 25, 115-123.

Jaenisch, R (**1976**). Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. **Proc. Natl. Acad. Sci.** 73, 1260-1264.

Jahner, D and Jaenisch, R (**1985**) Retrovirus-induced *de novo* methylation of flanking host sequences correlates with gene inactivity. ***Nature*** 315, 594-597.

Jahner, D, Stuhlmann, H, Stewart, CL, Harbers, K, Lohler, J, Simon, I and Jaenisch, R (**1982**) *De novo* methylation and expression of retroviral genomes during mouse embryogenesis. **Nature** 298, 623-628.

Jang, SK, Krausslich, H-G, Nicklin, MJH, Duke, GM, Palmenberg, AC, and Wimmer, E (**1988**) A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during *in vitro* translation. ***J. Virol.*** 62, 2636-2643.

Keshet, E, Shaul, Y, Kaminchik, J, and Aviv, H (**1980**) Heterogeneity of 'virus-like' genes encoding retrovirus-associated 30S RNA and their organization within the mouse genome. **Cell** 20, 431-439.

Lacy E, Roberts S, Evans EP, Burtenshaw MD, and Constantini, FD (**1983**) A foreign beta-globin gene in transgenic mice: integration at abnormal chromosomal positions and expression in inappropriate tissues. ***Cell*** 34, 343-358.

Lenormand, P, Pribnow, D, Rodland, KD., and Magun, BE (**1992**) Identification of a novel enhancer element mediating calcium-dependent induction of gene expression in response to either epidermal growth factor or activation of protein kinase C. **Mol. Cell. Biol.** 12, 2793-2803.

Linney, E, Davis, B, Overhauser, J, Chao, E, and Fan, H (**1984**) Non-function of a Moloney murine leukaemia virus regulatory sequence in F9 embryonal carcinoma cells. **Nature** 308, 470-472.

Loh, TP, Sievert, LL, and Scott, RW (**1987**) Proviral sequences that restrict retroviral expression in mouse embryonal carcinoma cells. **Mol. Cell. Biol.** 7, 3775-3784.

Loh, TP, Sievert, LL, and Scott, RW (**1988**) Negative regulation of retrovirus expression in embryonal carcinoma cells mediated by an intragenic domain. **J. Virol.** 62, 4086-4095.

Matsuda, Y, Harada, Y-N., Natsuume-Sakai, S, Lee, K, Shiomi, T, and Chapman, V (**1992**) M. Location of the mouse complement factor H gene (*cfh*) by FISH analysis and replication R- lines is associated with methylation and dependent on its chromosomal position. **Cytogen. Cell. Genet.** 61, 282-285.

Nilsson, M, and Bohm, S (**1994**) Inducible and cell type-specific expression of VL30 U3 subgroups correlate with their enhancer design. **J. Virol.** 68, 276-288.

Norton, JD, and Hogan, BLM (**1988**) Temporal and tissue-specific expression of distinct retrovirus-like (VL30) elements during mouse development. ***Devel. Biol.*** 125, 226-228.

Ohnishi, K, Takagi, M, Kurokawa, Y, Satomi, S, and Konttinen, YT (**1998**) Matrix metalloproteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema. **Lab. Invest.** 78, 1077-1087.

Palmiter RD, Chen HY, and Brinster, RL (**1982**) Differential regulation of metallothionine-thymidine kinase fusion genes in transgenic mice and their offspring. **Cell** 29, 701-710.

Petersen, R, Kempler, G, and Barklis, E (**1991**) A stem cell-specific silencer in the primer-binding site of a retrovirus. ***Mol. Cell. Biol.*** 11, 1214-1221.

Price, J, Turner, D, and Cepko, C (**1987**) Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. **Proc. Natl. Acad. Sci. USA** 84, 156-160.

Robertson, EJ, ed. Teratocarcinomas and embryonic stem cells: a practical approach. **1987**. pp 71-151, IRL Press Limited, Oxford.

Rodland, KD, Jue, S F, and Magun, BE (**1986**). Regulation of VL30 gene expression by activators of protein kinase C. **J. Biol. Chem.** 261, 5029-5033.

Rodland, KD, Muldoon, LL, Dinh, T-H, and Magun, BE (**1988**) Independent transcriptional regulation of a single VL30 element by epidermal growth factor and activators of protein kinase C. **Mol. Cell. Biol.** 8, 2247-2250.

Sanes, JR, Rubenstein, JLR, Nicolas, J-F (**1986**) Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. **EMBO J.** 5, 3133-3142.

Scolnick, EM, Vass, WC, Howk, RS, and Duesberg, PH (**1979**) Defective retrovirus-like 30S RNA species of rat and mouse cells are infectious if packaged by type c helper virus. **J. Virol.** 29, 964-972.

Si-Hoe, SL, and Murphy, D. (**1999**) Production of transgenic rodents by the microinjection of cloned DNA into fertilized one-cell eggs. **Methods Mol. Biol.** 97, 61-100.

Takashi, E, Hori, T, O'Connell, PO, Leppert, M, and White, R (**1991**) Mapping of the MYC gene to band 8p24.12-q24.13 by R-banding and distal to fra(8)(q24.11), FRA8E, by fluorescence *in situ* hybridization. **Cytogen Cell Genet.** 57, 109-111.

Treisman, J, Hwu, P, Minamoto, S, Shafer, GE, Cowherd, R, Morgan, RA, and Rosenberg, SA (**1995**) Interleukin-2-transduced lymphocytes grow in an autocrine fashion and remain responsive to antigen. **Blood** 85, 139-145.

Vorbroker, DK, Profitt, SA, Nogee, LM and Whitsett, JA (**1995**) Aberrant processing of surfactant protein C (SP-C) in hereditary SP-B deficiency. **Am. J. Physiol.** 268, 647-656.

Watkins, S, (1989) *in* **Current Protocols in Molecular biology** (Ausubel, R. M., Brent, R., Kingston, R., Moore, D., Seidman, J. G., Smith, J. A., and Struhl, K., eds.) 1989. pp. 14.6.1-14.6.13, Wiley and Sons, Inc., New York, NY.